PROPERTIES AND SYNTHESIS OF THE RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE LARGE SUBUNIT BINDING PROTEIN

CATHERINE RUTH MORGAN (née Lennox) B.Sc. (Edinburgh)

Thesis submitted for the degree of Doctor of Philosophy.

University of Warwick,

Department of Biological Sciences.

DECEMBER 1985

CONTENTS

SUMMARY 1

ACKNOWLEDGEMENTS 2

DECLARATION 3

ABBREVIATIONS 4

SECTION ONE - INTRODUCTION 6

1.1) RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE 7

1.1.1) Historical background and significance 7

1.1.2) The structure of RUBISCO 9

1.1.3) Subunit synthesis 15

1.1.4) Enzymic properties 30

1.2) PHOTOREGULATION OF RUBISCO SYNTHESIS 37

1.2.1) Photoregulation of gene expression 37

1.2.2) Photoregulation of RUBISCO synthesis 47

1.3) THE RUBISCO LARGE SUBUNIT BINDING PROTEIN 54

1.3.1) Historical background 54

1.3.2) Possible roles of the binding protein 59

1.4) AIMS OF THE PROJECT 61

SECTION TWO - MATERIALS AND METHODS 63

2.1) GROWTH OF PLANTS 64

2.2) EXTRACTION OF TOTAL SOLUBLE PROTEINS
FROM PEA APICES 64

2.3) CHLOROPLAST ISOLATION 65
2.3.1) Isolation technique 65
2.3.2) Radiolabelling of chloroplast proteins 66
2.3.3) Synthesis of chloroplast proteins
in chloroplast stromal extracts 68

2.4) PROTEIN PURIFICATION 68

2.5) IMMUNOLOGICAL TECHNIQUES 72
2.5.1) Production and purification of antibodies 72
2.5.2) Preparation of iodinated protein A 75
2.5.3) Analysis of proteins by
immunoblotting ("Western" blotting) 76
2.5.4) Analysis of proteins by
rocket immunoelectrophoresis 77
2.5.5) Ouchterlony immunodiffusion 79

2.6) ANALYSIS OF PROTEINS BY
POLYACRYLAMIDE GEL ELECTROPHORESIS 80
2.6.1) Electrophoresis under denaturing
conditions 80

2.6.2) Electrophoresis under non-denaturing conditions 82
2.6.3) Staining of polyacrylamide gels 84
2.6.4) Electroelution of proteins from polyacrylamide gels 86
2.6.5) Determination of radioactivity in proteins subjected to non-denaturing electrophoresis ... 86

2.7) GENERAL TECHNIQUES 87
2.7.1) Protein assay 87
2.7.2) Chlorophyll assay 87
2.7.3) RUBISCO assay 88
2.7.4) Sucrose density gradient centrifugation 89
2.7.5) Autoradiography 89
2.7.6) Photography 90

2.8) CHEMICALS 90

SECTION THREE - RESULTS AND DISCUSSION 92

3.1) CHARACTERISATION OF THE RUBISCO LSU-BINDING PROTEIN 93
3.1.1) Radiolabelling of chloroplast proteins 93
3.1.2) The association of binding protein with newly-synthesised large subunits of RUBISCO ... 95

3.2) PURIFICATION AND PROPERTIES OF THE
RUBISCO LSU-BINDING PROTEIN COMPLEX 103
3.2.1) Purification of RUBISCO and the
RUBISCO LSU-binding protein complex 103
3.2.2) Purification and characterisation
of antibodies 111
3.2.3) The subunit molecular weight of the
LSU-binding protein 129
3.2.4) Reversible dissociation of the
RUBISCO LSU-binding protein complex 132
3.2.5) Discussion 144

3.3) PHOTOREGULATION OF THE LSU-BINDING PROTEIN
AND RUBISCO 146
3.3.1) Light-stimulated accumulation of
RUBISCO and the LSU-binding protein 146
3.3.2) Synthesis of the subunits of RUBISCO
on illumination of etiolated plants 153
3.3.3) Activity of RUBISCO in etiolated and
light-grown plants 163
3.3.4) Discussion 167

SECTION FOUR - CONCLUSIONS 170

4.1) PROPERTIES OF THE LSU-BINDING PROTEIN 171

4.2) SYNTHESIS OF THE LSU-BINDING PROTEIN 174

4.3) ROLES OF THE LSU-BINDING PROTEIN 177

SECTION FIVE - REFERENCES 183

APPENDIX ONE - PUBLICATIONS 218

LIST OF TABLES

3.1) Comparison of RUBISCO and RUBISCO LSU-binding protein contents in light-grown and dark-grown pea plants 154

LIST OF FIGURES

3.1) Incorporation of [^{35}S]-methionine into chloroplast proteins 94

3.2) Labelling of RUBISCO and RUBISCO large subunit-binding protein complex *in vitro* 96

3.3) The association of newly-synthesised LSUs of RUBISCO with RUBISCO large subunit-binding protein 98

3.4) Assembly of RUBISCO large subunit into holoenzyme 101

3.5) Elution profile from the Sephacryl 300 column illustrated by absorbance at 280nm 105

3.6) Elution profile from the Sephacryl 300 column illustrated by SDS polyacrylamide gel electrophoresis 106

3.7) Purification of RUBISCO and RUBISCO large subunit-binding protein complex illustrated by non-denaturing electrophoresis 108

3.8) Purification of RUBISCO and RUBISCO large subunit-binding protein complex illustrated by electrophoresis under denaturing conditions 109

3.9) Purity of preparations of RUBISCO and RUBISCO large subunit-binding protein complex 110

3.10) Characterisation of antibodies raised against the RUBISCO large subunit-binding protein complex as determined by Ouchterlony immunodiffusion 114

3.11) Characterisation of antiserum raised against RUBISCO large subunit-binding protein complex as determined by "Western" blotting 115

3.12) Characterisation of antiserum raised against RUBISCO as determined by "Western" blotting 116

3.13) Quantitation of immunodetection by "Western" blots 120

3.14) Quantitation of immunodetection of RUBISCO by "Western" blotting 121

3.15) Variability in immunodetection by "Western" blotting 122

3.16) Characterisation by rocket immunoelectrophoresis of antiserum raised against RUBISCO 125

3.17) Characterisation by rocket immunoelectrophoresis of antiserum raised against RUBISCO large subunit-binding protein complex 126

3.18) Quantitation of detection of RUBISCO by rocket immunoelectrophoresis 127

3.19) Quantitation of detection of
binding protein
by rocket immunoelectrophoresis 128

3.20) Determination of the
molecular weight
of the binding protein subunit 130

3.21) The effect of MgATP on
the RUBISCO LSU-binding
protein complex 134

3.22) Dissociation of the RUBISCO
LSU-binding protein complex by MgATP 135

3.23) The effect of ATP and GTP on the
RUBISCO LSU-binding protein complex 136

3.24) Analysis of stromal proteins
by sucrose density gradient
centrifugation 139

3.25) Analysis of stromal proteins
by sucrose density centrifugation
in the presence of ATP 140

3.26) Centrifugation profile of RUBISCO subunits and the RUBISCO LSU-binding protein 141

3.27) Centrifugation profile of RUBISCO subunits and the RUBISCO LSU-binding protein in the presence of ATP 142

3.28) Reversible dissociation of the RUBISCO LSU-binding protein complex 143

3.29) Increases in fresh weight and total soluble protein on illumination of etiolated Pisum sativum plants 149

3.30) Accumulation of chlorophyll on illumination of etiolated plants of Pisum sativum..... 150

3.31) Light-stimulated accumulation of RUBISCO and RUBISCO LSU-binding protein 152

3.32) Accumulation of RUBISCO subunits on illumination of pea plants as determined by silver staining 156

3.33) Accumulation of RUBISCO subunits
and RUBISCO LSU-binding protein on
illumination of etiolated plants as
determined by "Western" blotting 157

3.34) RUBISCO SSU is present in
etiolated pea plants 159

3.35) Limits of detection of RUBISCO subunits
by silver staining 160

3.36) Photoregulation of the RUBISCO
subunits and the RUBISCO LSU-binding protein ... 161

3.37) The presence of RUBISCO holoenzyme
in extracts from etiolated plants 164

3.38) The dependence of RUBISCO activity on
the amount of stromal protein present 165

3.39) The dependence of RUBISCO activity on
the concentration of RuBP present 166

SUMMARY

Previous work has shown that newly-synthesised and unassembled large subunits of the photosynthetic CO_2-fixing enzyme, ribulose-1,5-bisphosphate carboxylase (E.C. 4.1.1.39), are non-covalently associated with another chloroplast stromal protein, termed the carboxylase large subunit-binding protein. In the present work the large subunit-binding protein of Pisum sativum was shown to be present in stromal extracts both as a monomer of subunit molecular weight 59,000, and as an oligomeric complex with which unassembled large subunits are asssociated. The oligomeric complex is reversibly dissociated into monomers in the presence of MgATP; dissociation is nearly complete at 10mM MgATP, but concentrations as low as 0.1mM MgATP are sufficient to cause some dissociation.

The large subunit-binding protein complex and ribulose-1,5-bisphosphate carboxylase were purified from leaves of Pisum sativum and antibodies raised against these proteins were used to examine the photoregulation of accumulation of their subunits. On illumination of etiolated Pisum plants, an increase in the accumulation of all three subunits is observed. The small subunit of the carboxylase was shown to be the most strongly photoregulated of the three subunits. The abundance of small subunit increased 30-fold on illumination of an etiolated apex for 48 hours, while the same light treatment increased the amount of large subunit by 15-fold. The LSU-binding protein is readily detectable in etiolated plants, with amounts increasing three to four-fold on illumination. No close correlation between the accumulation of large subunit-binding protein and either of the carboxylase subunits was observed.

Both the small subunit and the large subunit of the carboxylase were found to be present in etiolated Pisum plants and these subunits are present in the form of the holoenzyme. The holoenzyme present in etiolated plants is catalytically active and possesses a specific activity which is the same as that measured in light-grown plants.

These data are discussed with reference to the hypothesis that the large subunit-binding protein is involved in the assembly of the carboxylase from its subunits.

ACKNOWLEDGEMENTS

I wish to acknowledge the following people for their assistance throughout the course of this work. My grateful thanks go first of all to Prof. John Ellis for all his suggestions and for his help with this thesis. Thanks are also due to Richard Johnson for his encouragement and for valuable discussion. I must also acknowledge the assistance of Mrs. J.E. Musgrove in the preparation of Figures 3.23 and 3.28.

I dedicate this thesis to my family and to Philip in gratitude for all their support over the past three years.

DECLARATION

I declare that all of the work in this thesis, except where specifically stated, was original research performed by myself under the supervision of Prof. R.J. Ellis and that none of this work has previously been submitted for any degree. All sources of information have been acknowledged by means of references.

Ruth Morgan.

RUTH MORGAN.

LIST OF ABBREVIATIONS

A	Absorbance
APS	Ammonium persulphate
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumin
Ci	Curie (3.7×10^{10} disintegrations per second)
cDNA	Complementary DNA
cpm	Counts per minute
ctDNA	Chloroplast DNA
Da	Daltons
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Einstein (mole of photons)
EC	Enzyme commission number
EDTA	Ethylenediaminetetraacetic acid
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
kbp	Kilobase pairs
kDa	Kilodaltons
LHCP	Light harvesting chlorophyll a/b protein
LSU	Large subunit of RUBISCO
mA	Milliampere
Mr	Relative molecular mass
mRNA	Messenger RNA

P20	Precursor to the SSU of RUBISCO
PBS	Phosphate buffered saline
pers. comm.	Personal communication
polyA$^+$	Polyadenylated
PMSF	Phenylmethylsulphonyl fluoride
RIE	Rocket immunoelectrophoresis
RNA	Ribonucleic acid
RUBISCO	Ribulose-1,5-bisphosphate carboxylase-oxygenase (EC 4.1.1.39)
RuBP	Ribulose-1,5-bisphosphate
SDS	Sodium dodecyl sulphate
SSU	Small subunit of RUBISCO
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylenediamine
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
tRNA	Transfer RNA
v/v	concentration by volume
w/v	concentration by weight

note: all polyacrylamide gel concentrations given as a percentage are expressed in terms of w/v.

SECTION ONE - INTRODUCTION

1.1 RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE

1.1.1 Historical background and significance

Van Helmont (?1577-1644) was the first to disprove the idea originating with Aristotle that plant material was synthesised exclusively from soil. His experiment was elegant in its simplicity: "I took an earthen vessel in which I put 200 pounds of soil dried in an oven, then I moistened with rain-water and pressed hard into it a shoot of willow weighing 5 pounds. After exactly five years the tree that had grown up weighed exactly 169 pounds and about 3 ounces. But the vessel had never received anything but rain-water or distilled water to moisten the soil when this was necessary, and it remained full of soil, which was still tightly packed, and, lest any dust from outside should get into the soil, it was covered with a sheet of iron with tin perforated with many holes. I did not take the weight of the leaves that fell in the autumn. In the end I dried the soil once more and got the same 200 pounds that I started with, less about 2 ounces. Therefore the 164 pounds of wood, bark and root arose from the water alone"

(from Fogg, 1976)

It was not until 1727 that Hales suggested that plants were "very probably drawing some part of their nourishment from the air" (from Fogg, 1976). Priestley in 1772 demonstrated that plants could give off oxygen and Ingen-housz in 1779 showed that light was essential for this process. It was

Senebier in 1782 who completed the picture by showing that the presence of CO_2 was necessary for the production of oxygen in the light. The concept of photosynthesis was therefore conceived 170 years before Benson and Calvin in the 1950's elucidated the biochemical pathways involved in the fixation of CO_2 and its consequent appearance in plant biomass (Fogg, 1976).

On land, the seed plants are the principal agents of photosynthesis with the algae playing a minor part; both groups in total fix 1.6×10^{10} tons of carbon per year. In the oceans the unicellular photosynthetic organisms contribute 1.2×10^{10} tons of fixed carbon per year. The immense importance of carbon fixation in carbon cycling becomes clear when it is appreciated that the total CO_2 of the atmosphere would be exhausted in less than 20 years if it were not replenished by the processes of decomposition and respiration (Stanier *et al.*, 1978).

The Calvin cycle is the sole pathway in eukaryotes by which net reductive CO_2 fixation takes place and the key step in this pathway is the formation of two molecules of 3-phosphoglycerate from ribulose bisphosphate, CO_2 and H_2O. The enzyme catalysing this reaction was first purified by Wildman & Bonner (1947) and termed Fraction 1 protein because of its sedimentation properties. The first direct evidence that this protein was the carboxylation enzyme came from Weissbach *et al.* (1956). These workers isolated and partially purified the enzyme from spinach (*Spinacia oleracea*) and showed that the enzymic activity

responsible for the formation of phosphoglycerate from CO_2 and ribulose bisphosphate was associated with a protein which had a sedimentation coefficient of 18s. Over the past thirty years this enzyme has been variously called Fraction 1 protein, ribulose bisphosphate carboxylase and 3-phospho-D-glycerate carboxy-lyase(dimerising). The problem of nomenclature worsened when in 1971 it was discovered that the protein could also catalyse an oxygenation reaction, resulting in the formation of one molecule each of 3-phosphoglycerate and 2-phosphoglycolate from ribulose bisphosphate and molecular oxygen (Bowes et al., 1971). This discovery necessitated the introduction of the title ribulose-1,5-bisphosphate carboxylase-oxygenase which has subsequently been replaced with the acronym RUBISCO by many workers.

RUBISCO is the most abundant protein in the world (Ellis, 1979); it has been calculated that 40 million tons of it are required to support the rates of photosynthesis discussed above. RUBISCO is a very complex protein with respect to its enzymic and physicochemical properties. These features coupled with its abundance and the central role it plays in carbon fixation have led to a continuing interest in all aspects of the enzyme.

1.1.2 The structure of RUBISCO

RUBISCO has been identified in extracts from a variety of prokaryotic microorganisms, algae and higher plants (McFadden & Purohit, 1978; McFadden, 1980; Johal &

Chollet, 1980; Miziorko & Lorimer, 1983). The enzyme is an oligomeric protein and the RUBISCO of all algae and higher plants has a hexadecameric structure comprising eight large subunits (LSU) and eight small subunits (SSU). The subunits have relative molecular masses in the ranges of 51,000 to 58,000 and 12,000 to 18,000 respectively. The oligomer therefore has a relative molecular mass of between 500,000 and 600,000 in all higher organisms. Electron microscopic studies of Chinese cabbage RUBISCO in 1965 first revealed the complex quaternary structure of the enzyme (Haselkorn et al., 1965) and subsequently there have been numerous investigations on this aspect of the enzyme, including X-ray crystallographic studies (reviewed by Miziorko & Lorimer, 1983). These have shown that the plant enzymes all possess a similar eight LSU plus eight SSU (L_8S_8) structure but the way in which the sixteen subunits are arranged is unclear, although a number of models have been proposed (Miziorko & Lorimer, 1983; Roy et al., 1978a; Gray & Kekwick, 1974a)

The structure of bacterial RUBISCO has been reported to display much variability in subunit composition and molecular weight (McFadden, 1980). Early investigations into the quaternary structure of bacterial enzymes led to reports of L_4 and L_8 enzymes (McFadden, 1980). The ease with which mild acid treatment will remove SSU during purification (Andrews & Abel, 1981) casts doubt upon the natural occurrence of these oligomeric LSU enzymes. More recent studies suggest that there exist three different forms

of RUBISCO in prokaryotes and that in those bacteria which utilise the Calvin cycle to fix CO_2 it is the L_8S_8 form which is commonest (Gatenby et al., 1985). The hexadecameric prokaryotic RUBISCO appears to have a similar subunit arrangement to that observed in the higher plant enzyme. For example, in Alcaligenes eutrophus a bilayer is present; each layer is composed of four LSU molecules (Bowien et al., 1980). As with the plant enzyme the molecule has a four-fold rotational symmetry, possesses a central pore, and is organised in layers which are arranged perpendicularly to the axis of symmetry.

In addition to the standard L_8S_8 enzyme, some members of the Rhodospirillaceae possess RUBISCOs with different structures. The RUBISCO in Rhodospirillum rubrum is dimeric (Tabita & McFadden, 1974) and has a molecular weight of 114,000. It is composed of subunits which have a molecular weight similar to that of LSU found in higher plants. A second enzyme type consisting only of LSU has been reported in some other members of the Rhodospirillaceae. Rhodopseudomonas capsulata and Rhodopseudomonas sphaeroides possess hexameric RUBISCO of molecular weight 360,000 (Gibson & Tabita, 1977a,b). The enzyme of the latter organism has been shown to lack SSU and to possess LSU of Mr 52,000 (Muller et al., 1985). Rhodopseudomonas blastica has a similar hexameric enzyme (Sani et al., 1983).

The eukaryotic LSU is thought to be synthesised as a larger precursor. This has been detected in B. oleracea (Langridge, 1981) and in Zea mays (Gatenby, 1984). The

precursor has a Mr which is 2,000 larger than the mature form. It has been found by DNA sequencing techniques that in the LSU of Chlamydomonas reinhardtii there must be a methionine residue at the N-terminus when the gene is expressed (Dron et al., 1982). A similar situation is seen with the LSU of both Z. mays and S. oleracea. However, the LSUs of purified barley and wheat RUBISCOs possess an alanine residue at the N-terminus (Miziorko & Lorimer, 1983). The gene sequences of Z. mays, S. oleracea and C. reinhardtii RUBISCO LSU all encode an alanine residue at position 15 and therefore it is likely that the first 14 residues are removed after translation. Whether this occurs before or during purification of the enzyme is unclear.

An important feature of isolated higher plant LSU is its relative insolubility in aqueous media. The LSU can be separated from SSU using urea, SDS, and extremes of pH (Jensen & Bahr, 1977). If urea is used to dissociate the subunits, on subsequent removal of urea by dialysis an insoluble aggregation of LSU results (Voordouw et al., 1984). Similarly, if RUBISCO-containing solutions from S. oleracea are taken to pH 5.2 to remove SSU then LSU is found to precipitate (J. Andrews, pers. comm.). It has also been shown that newly-synthesised LSU of Z. mays expressed in E. coli is present in an insoluble form in the absence of SSU, suggesting that either SSU or some other factor is necessary to maintain solubility of higher plant LSU (Gatenby, 1984). All of these insoluble forms of higher plant LSU are enzymically inactive.

In contrast, the RUBISCO from cyanobacterial sources remains soluble even in the complete absence of SSU (Andrews & Abel, 1981). This is surprising in view of the homologies between the amino-acid sequences of higher plant and cyanobacterial LSUs. Recently the presence of a soluble and enzymically active L_8 form of RUBISCO in Chromatium vinosum has been reported (Torres-Ruiz & McFadden, 1985). The major difference between prokaryotes and eukaryotes is that in the latter the two subunits are synthesised in different cellular compartments and it is therefore possible that the mode of interaction between the two subunits in the assembly reaction in eukaryotes and prokaryotes is different. If so, this may help in explaining these differences in solubility.

Cloning the eukaryotic SSU gene has allowed the sequencing of the precursor to the mature protein; the SSU precursor was first identified in Chlamydomonas reinhardtii and found to be 3,500Da larger than the mature form (Dobberstein et al., 1977). Such a precursor was subsequently identified in P. sativum (Chua & Schmidt, 1978; Highfield & Ellis, 1978). The precursor to the SSU of Chlamydomonas reinhardtii has been sequenced and found to have an N-terminal extension of 44 amino-acids (Schmidt et al., 1979). This extension sequence possesses an abundance of valine, serine and proline residues and overall has a greater number of basic than acidic residues. The SSU precursor of Glycine max has 178 amino-acids, 55 of which are in the N-terminal extension (Berry-Lowe et al., 1982).

The *Glycine max* and *Chlamydomonas reinhardtii* N-terminal extension sequences lack homology but both contain many basic amino-acids. In contrast, the *P. sativum* and *G. max* extension sequences possess much homology (Berry-Lowe *et al.*, 1982). The mature SSU of *P. sativum* has a lower isoelectric point than that of the precursor (Ishiye *et al.*, 1981). Although the SSU precursor is nuclear-encoded and synthesised in the cytoplasm, RUBISCO assembly occurs in the chloroplast stroma after transport of the precursor across the chloroplast envelope. The amino-acid composition of the extension peptide may confer a positive charge on the SSU to allow interaction with the negatively-charged chloroplast envelope prior to import.

The lack of homology between the *Chlamydomonas* and higher plant extension sequences, and the finding that higher plant chloroplasts could not import the algal SSU, led workers to believe that there had been much evolutionary divergence in the extension sequences (Chua & Schmidt, 1978). Subsequent studies have shown that the SSU precursor of *Chlamydomonas* can be taken up by vascular plant chloroplasts but is processed by the latter to an intermediate form at a site which is conserved in the extension sequences of algal and vascular plant precursors (Mishkind *et al.*, 1985). This observation shows that the features of the molecule essential for function have to an extent been retained. The steps involved in the import and processing of the SSU precursor will be discussed more fully in the next section.

1.1.3 Subunit synthesis

Initially I intend to discuss the synthesis of the eukaryotic RUBISCO subunits, as it is in such organisms that the synthesis is most complex; the possession of the chloroplast and the resulting compartmentation of the cell contribute to this complexity. The synthesis of bacterial RUBISCO subunits will be discussed at the end of this section.

The presence of both DNA and RNA in chloroplasts was established in the 1960's. In 1962 Ris and Plaut located DNAse-sensitive fibrils within the chloroplasts of Chlamydomonas using uranyl acetate fixation techniques in electron microscopy (Kirk & Tilney-Bassett, 1978). Higher plant chloroplasts have also been shown to contain DNA and this differs from that of the nucleus in degree and rate of renaturation (Hermann, 1972). The physical properties of chloroplast DNA (ct DNA) have been reviewed by Bedbrook & Kolodner (1979).

Chloroplasts have been shown to possess DNA polymerase, RNA polymerase and ribosomes (reviewed by Boulter et al., 1972). The chloroplasts of higher plants contain ribosomes of similar size and character to those of prokaryotes (Lyttleton, 1962; Ellis, 1969; Ellis, 1970; Meaker & Tewari, 1980), a fact of which use has been made in determining the sites of synthesis of chloroplast proteins (see below). Chloroplast ribosomes can constitute as much as

30% of the total ribosomes of the cell (Bedbrook et al., 1977) and, therefore, chloroplasts it would seem, have a considerable capacity for protein synthesis. However, analysis of proteins present within the stromal compartment and thylakoids show that the ctDNA is not capable of encoding all of the chloroplast proteins (Ellis, 1981). A two-dimensional polyacrylamide gel analysis of chloroplast proteins allows a resolution of about 190 polypeptides, and a consideration of the total molecular weight of these has shown that the chloroplast DNA is not capable of encoding such a total molecular weight of polypeptide. It has been estimated from a consideration of the metabolic complexity of the chloroplast, that there may well be as many as 1000 polypeptides required within the organelle (Dyer, 1984). There must therefore be a continual and considerable interaction of both chloroplast and nuclear genomes with respect to proteins and the myriad of metabolites essential for chloroplast function. The evidence that the majority of chloroplast proteins are nuclear in origin has come from using a variety of techniques and these will be discussed below, with special reference to RUBISCO.

The first experiments designed to localise the coding sites of the LSU and SSU of RUBISCO made use of the fact that chloroplast ribosomal activity can be inhibited by chloramphenicol, lincomycin and spectinomycin, inhibitors of bacterial ribosomes (Margulies, 1971; Ellis & Hartley, 1971). Chloramphenicol has been shown to specifically inhibit the *in vivo* synthesis of the higher plant LSU, while

cycloheximide, an inhibitor of protein synthesis by 80s ribosomes, will inhibit synthesis of SSU (Criddle et al., 1970). High concentrations of cycloheximide have also been found to inhibit LSU synthesis however. Margulies (1971) used chloramphenicol to study protein synthesis in Chlamydomonas reinhardtii. In these studies synthesis of RUBISCO was inhibited not only by the 70s ribosome inhibitor but also by cycloheximide, suggesting that both chloroplast and cytoplasm participate in RUBISCO synthesis.

Another inhibitor of 80s ribosomes is MDMP (2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide) and this was found to inhibit synthesis of both LSU and SSU if applied to intact pea leaves (Ellis, 1975). This inhibitor does not affect 70s ribosomal protein synthesis and yet LSU synthesis is inhibited. The author suggested that SSU may have been necessary as a positive initiation factor for synthesis of LSU at either the transcriptional or translational level, but it may equally well be the case that LSU synthesis occurs in the presence of MDMP but its accumulation in the chloroplast is inhibited since it cannot complex with SSU and is subsequently degraded.

These early inhibitor studies suggested that LSU was synthesised on chloroplast ribosomes while SSU was synthesised in the cytoplasm on 80s ribosomes, but interpretations of the data were made with reservations. Attention had been drawn to the finding that use of a single inhibitor could lead to incorrect interpretations of the data. Also some antibiotic inhibitors were found to have some

effect on systems other than protein synthesis (Ellis, 1977).

An indirect method which has shown SSU is synthesised on 80s ribosomes has been used by Feierabend and his coworkers. Leaves of a number of higher plants were found to be chlorotic and deficient in 70s ribosomes if plants were grown at temperatures between 28°C and 34°C (Feierabend & Mikus, 1977). It was found that RUBISCO was completely absent in these plants. A subsequent immunological study with heat-treated rye plants grown at 32°C showed that SSU was present in total plant extracts in the absence of LSU (Feierabend & Wildner, 1978).

Less disruptive methods have been employed to elucidate the sites of subunit synthesis. These have included immunological studies and the use of cell-free transcription and/ or translation systems. In the former, antibody raised against Phaseolus vulgaris SSU was found to preferentially precipitate 80s ribosomes which were therefore concluded to be synthesising the nascent SSU polypeptide (Gray & Kekwick, 1974b).

A much more direct approach to the problem has been employed in more recent studies utilising intact chloroplasts isolated from higher plants. The first such in vitro studies were performed with Pisum chloroplasts (Blair & Ellis, 1973; Ellis, 1977). In this system, chloroplasts are incubated in the presence of radiolabelled amino-acids in the light with photosynthetically-generated ATP driving chloroplast protein synthesis. No exogenous ATP need be added and this means that the chloroplast preparation need not be

very pure: contamination by mitochondrial and nuclear DNA is of no importance, although care should be taken to ensure that there is no bacterial contamination. The major radiolabelled product from the stromal compartment of chloroplasts incubated in this way is the LSU of RUBISCO (Blair & Ellis, 1973). Heterologous cell-free protein synthesising systems have also been employed: Spinacia chloroplast total RNA added to an extract of E. coli will synthesise LSU (Hartley et al., 1975). Roy et al., (1976) found that Triticum leaf cytoplasmic ribosomes will synthesise SSU if Triticum seedling polysomes are translated in a wheat germ translation system.

An early method of determining the sites of encoding of the RUBISCO subunits was the genetic approach of interspecific hybridisation. The studies were based mainly on Nicotiana species. In Nicotiana, it is only through the maternal line that chloroplast DNA can be inherited. Reciprocal hybrids in which sources of male and female gametes are reversed have been useful in providing information on the coding site of RUBISCO subunits. The genes encoding Nicotiana LSU have been found to be inherited only through the maternal line and therefore are located in ctDNA (Sakano et al., 1974). Such studies with Nicotiana were possible because a number of isoelectric variants of LSU exist among species and these serve as phenotypic markers for ctDNA genes (Sakano et al., 1974). Corroborant evidence that the chloroplast genome is the coding site for LSU came from the finding that a non-Mendelian mutation affects the

structure and function of the LSU in Chlamydomonas (Spreitzer & Mets, 1980). Further interspecific hybridisation studies by Kawashima & Wildman (1972), in which the inheritance of tryptic peptides of Nicotiana SSU was examined, have shown that these are inherited in a Mendelian fashion and that therefore SSU is encoded by nuclear DNA.

With the advent of gene cloning, the question of the subunit coding site has been resolved conclusively. The genes for both LSU and SSU have now been cloned for a number of higher plant and algal species and interesting data have emerged. The chloroplast genomes for a number of Nicotiana species have been extensively characterised (Zhu et al., 1982; 1984; Kung et al., 1982; Shen et al., 1982; Tassopulu & Kung, 1984) and the LSU gene has been shown to be located on the DNA circle. The LSU gene has also been cloned and characterised from Pisum sativum (Oishi & Tewari, 1983), Zea mays (Link et al., 1978; McIntosh et al., 1980), Chlamydomonas reinhardtii (Selvin et al., 1977; Dron et al., 1982), Euglena gracilis (Steigler et al., 1982) and Spinacia (Zurawski et al., 1981). Therefore there is now a substantial body of physical proof that the chloroplast genome is the coding site for LSU.

The Nicotiana LSU gene has been cloned and expressed in E. coli and the Chlamydomonas LSU gene cloned and expressed in B. subtilis (Zhu et al., 1984). The LSU genes from Zea mays and Triticum have also been expressed in E. coli (Gatenby et al., 1981) and the LSU made by the former is larger than the mature form suggesting

that it is synthesised as a precursor (Gatenby, 1984). This supports the earlier finding of Langridge (1981) which was described in Section 1.1.2. The newly-synthesised Zea mays LSU was found to be insoluble in the E. coli extract suggesting that some chloroplast factor absent in E. coli is necessary to maintain solubility of LSU prior to its assembly into RUBISCO holoenzyme (Gatenby, 1984).

Chloroplast DNA is interesting since in some species it possesses two large segments which are inverted repeats of each another. These regions of DNA contain the genes for chloroplast rRNA and it is thought that the inverted repeats may maintain ctDNA stability (Dyer, 1984). The Zea mays ctDNA has been extensively characterised (Bedbrook & Bogorad, 1976; Bedbrook et al., 1977). The two single copy regions which separate the inverted repeats are 18,500bp and 106,000bp long. It is in the larger of the two single copy regions that the gene for the LSU of Zea mays is located. Legumes differ from this in ctDNA structure. Pisum sativum ctDNA has no inverted repeat and there is a subsequent reduction in ctDNA size. Characterisation of the eukaryotic chloroplast genome shows that there is only one copy of the LSU gene per ctDNA circle (Dyer, 1984). Recently, however, it has been found in Zea mays that a single gene for the LSU can give rise to two different transcripts which differ in the length of their 5' untranslated region (Crossland et al., 1984). Control of LSU gene expression may therefore prove to be rather complex. The consequences of this will be discussed below in a consideration of subunit stoichiometry

and coordination.

RUBISCO SSU is nuclear encoded; isolated pea nuclei will synthesise transcripts of SSU which can be detected with cloned DNA probes (Gallagher & Ellis, 1982). A multigene family encodes the SSU. A number of members of this have been characterised in *Glycine max* (Berry-Lowe *et al.*, 1982) and *Petunia* (Dunsmuir *et al.*, 1983). In *Glycine max* a multigene family of at least ten members encodes the SSU of RUBISCO and in *Petunia* there appear to be at least four and no more than twelve SSU genes transcribed in leaf tissue. The cDNAs encoding SSU sequences from *Pisum sativum* have been cloned and sequenced (Bedbrook *et al.*, 1980; Coruzzi *et al.*, 1983).

Analyses of SSU gene sequences have confirmed that SSU is synthesised as a precursor (see Section 1.1.2). In *Glycine max* one member of the SSU gene family has been characterised and found to possess 3 exons and 2 introns. The sequence encoding the the extension peptide was found to comprise all of exon 1; the first 2 amino-acids of the mature polypeptide were also present in this exon (Berry-Lowe *et al.*, 1982). The *Chlamydomonas* precursor can be processed to the mature form by an endoprotease present in algal extracts. Precursors were subsequently found for SSU in *Spinacia* and *Pisum* (Chua & Schmidt, 1978; Highfield & Ellis, 1978) by programming a wheat germ translation system with poly A^+ RNA and analysing the products by immunoprecipitation. In *Pisum* the precursor has a Mr of 20,000 and similarly in *Spinacia* the precursor has a

relative molecular mass which is 4,000 - 5,000 larger than the mature form.

It is evident from the above that the sequence of events leading to the synthesis of a mature SSU capable of assembly into RUBISCO holoenzyme is complex. For assembly to proceed the SSU must enter the chloroplast stromal compartment by crossing the chloroplast envelope and be processed to the mature form. I shall now consider the evidence pertinent to these aspects of SSU synthesis.

It has been demonstrated that the SSU precursor is imported into the chloroplast: Pisum sativum SSU precursor is taken up by isolated chloroplasts and can be shown to be resistant to trypsin treatment (Highfield & Ellis, 1978). The uptake of the SSU precursor does not require concomitant protein synthesis and indeed has been shown to proceed in the absence of 80s ribosomes (Highfield & Ellis, 1978). This latter finding therefore distinguishes post-translational uptake of proteins by chloroplasts from that seen in microsomes where uptake is co-translational involving bound ribosomes (Blobel & Dobberstein, 1975). A further difference exists between co-translational protein transport and the post-translational uptake of the SSU precursor into chloroplasts. In the former the protein for export is synthesised as a higher molecular weight form, possessing an N-terminal signal sequence which is rich in hydrophobic amino-acids. Few acidic amino-acids are present in the signal sequence (Burstein & Schechter, 1977). It is thought that signal hydrophobicity allows insertion into and

passage through the membrane by association with membrane proteins. In contrast, the so-called N-terminal transit sequences involved in chloroplast protein precursor uptake have an amino-acid composition which renders them basic overall (Mishkind et al., 1985). The precursor therefore has a higher isoelectric point than does the mature form and this is thought to allow precursor interaction with the negatively-charged chloroplast envelope (Ishiye et al., 1981). To date there is no information available on how the SSU precursor actually traverses the chloroplast double membrane, although it has been suggested that the transit sequence may be involved in envelope recognition (Smith & Ellis, 1979; Ellis, 1981). There has been some circumstantial evidence presented which suggests that receptor proteins may be involved in the membrane passage and/or membrane recognition; chloroplasts incubated with proteases fail to import SSU precursor (Chua & Schmidt, 1978). More recent evidence has now been presented which shows that the transit sequence possesses all of the sequence information required for translocation of the SSU precursor into the chloroplast. Van den Broeck et al. (1985) constructed a chimaeric gene in which DNA encoding the transit sequence of one SSU polypeptide was fused to the gene which encodes bacterial neomycin phosphotransferase. The gene was introduced into Nicotiana cells and chloroplasts isolated from the resulting calli were found to contain the bacterial enzyme. The enzyme remained in the Nicotiana cell cytoplasm if the transit sequence was not present in the chimaeric gene.

Uptake of the SSU precursor is energy-dependent (Grossman et al., 1980). The energy requirement is met by ATP directly. Addition of ATP to chloroplast suspensions will substantially increase the import of the SSU precursor in the dark and ATP derived from photosynthetic phosphorylation will also stimulate import (Grossman et al., 1980).

Once inside the chloroplast the precursor is processed to the mature form within the stromal compartment (Smith & Ellis, 1979), and the processing activity has been partially purified from Pisum sativum chloroplasts (Robinson & Ellis, 1984a). This processing activity is soluble (Robinson & Ellis, 1984b). Processing of the precursor takes place in two stages via an intermediate and evidence suggests that a single enzyme may perform both cleavages (Robinson & Ellis, 1984b).

Vascular plant transit peptides for SSU have homologies in their amino-acid sequences. This may be the result of retention of a structure necessary for import. An interchangeable import of Pisum and Spinacia precursors into Pisum and Spinacia chloroplasts has been demonstrated (Chua & Schmidt, 1978). It has also been reported that Spinacia and Pisum chloroplasts will import the SSU precursor of Chlamydomonas (Mishkind et al., 1985), contrary to an earlier report (Chua & Schmidt, 1978). The later work shows that although the transit sequence of the algal SSU has diverged from those of the vascular plants in terms of sequence homology, import and a partial

processing can proceed: this processing occurs at a region which is conserved in the transit sequences of algal and vascular plant SSU precursors. The partially-processed algal SSU precursor can be converted to the mature form only by the algal transit peptidase (Mishkind *et al.*, 1985), showing that only one processing site has been conserved. The demonstration that the algal SSU precursor is processed in two steps in a way similar to the *Pisum* precursor suggests conservation of the process as a whole.

Precursors of *Pisum* and *Chlamydomonas* SSU can be assembled into holoenzyme once processed to the mature form in stromal extracts (Chua & Schmidt, 1978; Smith & Ellis, 1979; Mishkind *et al.*, 1985). This demonstrates that synthesis as a precursor and uptake by chloroplasts are physiological processes. The transit peptide may function in this assembly in some respect. The assembly reaction will be considered in detail in Section 1.3

In contrast to the complexity of the eukaryotic RUBISCO subunit synthesis, the prokaryotic system is simpler and is particularly well characterised in cyanobacteria. The LSU and SSU genes of the cyanobacterium *Anabaena* have been sequenced and the unique SSU gene is located downstream from the LSU gene. The SSU gene has no transit peptide sequence and there are no introns as are seen in eukaryotic systems. The SSU and LSU genes of *Anabaena* are co-transcribed (Nierzwicki-Bauer *et al.*, 1984). Similarly, in *Synechococcus* both LSU and SSU genes have been found to be close together on the DNA. Both polypeptides have been

expressed in E. coli (Gatenby et al., 1985).

The LSU gene of the Rs. rubrum enzyme and the LSU gene of the intermediate RUBISCO form from Rp. sphaeroides have been cloned (Somerville & Somerville, 1984; Muller et al., 1985). The latter is encoded by one gene only but this gene does hybridise to a second locus on the chromosome. It has been suggested that this second locus may represent an unexpresssed pseudogene or, more interestingly, may code for the LSU of the hexadecameric RUBISCO. The work of Shively et al. (1984) suggests that in Rp. capsulata two genes encoding LSU exist since the mobilities of the LSUs from the two forms differ on polyacrylamide gels. The work of Gibson & Tabita (1977c) shows that antiserum to the L_8 form of the enzyme fails to inhibit L_8S_8 enzyme activity, suggesting that the LSUs may indeed be different.

Finally, in this consideration of RUBISCO subunit synthesis, the problem of coordination of synthesis will be addressed. It is evident from the above that in eukaryotes the control of gene expression during subunit synthesis will be of paramount importance. In prokaryotes the situation is simpler: both LSU and SSU are encoded in the same cellular compartment and in the organisms studied to date the genes for the two subunits are co-transcribed. Also the gene copy numbers for prokaryote subunits appear to be similar. The situation in eukaryotes contrasts significantly with that of prokaryotes; in the former there must be cooperation between the nuclear and chloroplast genetic systems to ensure that both subunits come together in stoichiometric amounts in the

chloroplast stroma. The problem of coordinated synthesis arises because of the discrepancy in gene copy numbers for LSU and SSU. There is a vast excess of LSU genes over genes for SSU. A careful study has shown that there are approximately 300 copies of ctDNA per plastid and older leaf tissue can contain up to 150 chloroplasts per cell (Boffey & Leech, 1982). There are therefore upwards of 10,000 copies of ctDNA (and hence of LSU genes) per cell in contrast to between 6 and 12 copies of the SSU gene per cell (Berry-Lowe et al., 1982). For a coordinated synthesis to proceed there must be regulation at some stage in RUBISCO synthesis. It is unlikely that chloroplast gene expression will regulate the synthesis of RUBISCO; control is more likely to be exerted by the nucleus as it is the SSU gene copy number which is limiting. Some evidence does exist in support of the hypothesis that the nucleus controls subunit accumulation and this will be presented below.

Inhibitors of protein synthesis by 80s ribosomes can prevent accumulation of LSU in Pisum sativum (Ellis, 1975). It was therefore suggested that LSU synthesis may in some way be connected to that of SSU. Studies with a polyploid series of Triticum have shown that the amount of RUBISCO accumulation per cell increases with nuclear ploidy (Dean & Leech, 1982a). In the Triticum plants examined the ratio of RUBISCO amount per mesophyll cell to nuclear DNA was constant, suggesting nuclear control.

There exists conflicting evidence on the question as to how far the synthesis of SSU is coordinated with that of

LSU. In Glycine max leaf cells a continued synthesis of LSU can be detected in the absence of SSU synthesis (Barraclough & Ellis, 1979). Synthesis of LSU continued for 4 hours, suggesting that synthesis of both subunits was not stringently coupled. Also in this study the LSU formed was found to assemble into holoenzyme, showing that unassembled SSU was available. Indeed pools of unassembled SSU do appear to be present in Pisum leaves (Roy et al., 1978b), although these do have a short lifetime (see below). Lack of coordination of subunit synthesis is also seen in Glycine max cells in culture which have been subjected to a heat shock for one to two hours (Vierling & Key, 1985). It was found that this treatment led to a huge drop in SSU synthesis which was paralleled by a drop in mRNA levels for SSU. The LSU synthesis was not so drastically affected and there was little change in LSU message level. Therefore it appeared that coordination of LSU and SSU synthesis could be disrupted over a short time interval.

In contrast to the studies with Glycine max, a tight coordination of synthesis has been suggested in Triticum, Hordeum and Chlamydomonas (Dean & Leech, 1982b,; Nivison & Stocking, 1983; Iwanij et al., 1975). In all three studies synthesis of both subunits was monitored by the degree of incorporation of radiolabelled amino-acids and simultaneous changes in synthesis were observed in each case. However, in these three cases no attempt was made to examine turnover of newly synthesised subunits, the steady-state levels only being reported. It has been shown that a

mechanism does exist to digest unassembled SSU in chloroplasts of Chlamydomonas (Schmidt & Mishkind, 1983). These workers found that SSU has a short half-life in the chloroplast unless assembled into holoenzyme. It is interesting that the SSU protease detected in Chlamydomonas is nuclear-encoded, again suggesting that some control is exerted by the nucleus. The pools of SSU detected in Pisum leaves by Roy et al. (1978b) have subsequently been shown to have a short half-life of less than 4 minutes (Roy et al., 1979), suggesting that in higher plants also there may be a mechanism to regulate subunit stoichiometry by proteolysis.

1.1.4 Enzymic properties

RUBISCO catalyses the first reaction in both the photosynthetic and photorespiratory cycles. An early study of the kinetic constants for both reactions proved to be consistent with the finding that the same enzyme catalyses both reactions (Laing et al., 1974). Kinetic studies also have shown that the enzyme must undergo activation by CO_2 and Mg^{2+} to ensure maximum catalytic capability. The mechanism of activation was deduced for the Spinacia enzyme by Lorimer et al. (1976), and the activator CO_2 shown to bind to Lysine residue 201 of the LSU (Lorimer, 1981).

In the photosynthetic cycle, there results a net fixation of CO_2, while in the photorespiratory cycle a

net loss of carbon as CO_2 results. As the two cycles are interlocked through RUBISCO, it is the catalytic properties of the enzyme which determine the overall extent to which carbon is fixed. Currently, perhaps the most challenging aspects of RUBISCO biochemistry are the continued attempts to improve photosynthetic carbon fixation by altering the properties of the enzyme. To do so requires an understanding of the synthesis, assembly and catalytic properties of the RUBISCO. Some important features of the subunit synthesis have been described above in Section 1.1.3. The mechanisms of action of both the carboxylase and oxygenase activities have been clearly and fully reviewed elsewhere (Lane & Miziorko, 1979, Lorimer, 1981, Miziorko & Lorimer, 1983) and I do not wish to discuss these further. In this section I wish to consider the roles of both of the subunits in the RUBISCO-catalysed reactions and to discuss some of the attempts to improve photosynthetic carbon fixation.

Assigning roles to the subunits of RUBISCO has proved relatively easy for LSU and difficult for SSU. The activation and catalytic processes are a function of the LSU. Direct evidence that this is so comes from a consideration of the Rs. rubrum enzyme which is composed of only LSUs and undergoes activation by CO_2 and Mg^{2+} and will catalyse both carboxylase and oxygenase reactions (Christeller & Laing, 1978). The findings that the activator CO_2 is trapped on the lysine 201 of the LSU (Lorimer, 1981) and that affinity labelling will derivitise the lysine 175 of LSU (Hartman et al., 1978) support the above

statement. The divalent metal ion involved in activation binds to the LSU of both Spinacia and Rs. rubrum RUBISCO (Miziorko et al., 1982). In Rp. sphaeroides both forms of the enzyme can be activated even although only one form possesses SSU (Gibson & Tabita, 1979) and for the RUBISCO from Phaseolus vulgaris, antibody raised against the LSU will inhibit enzyme activity while antibody raised against the SSU has no effect (Gray & Kekwick, 1974b). This indirect evidence is consistent with the view that the active site is positioned on the LSU. Genetic evidence too has been produced: in Chlamydomonas mutants which lack RUBISCO activity the LSU has an altered isoelectric point (Spreitzer et al., 1982). Use has been made of specific affinity labels in determining the residues of LSU which are important in catalysis and activation. The lysine at position 201 has already been discussed. The lysines at positions 335 and 175 have been labelled in Spinacia and are thought to be present in the active site (Hartman et al., 1978). In the Rs. rubrum LSU the lysine residue corresponding to the eukaryotic lysine 175 is affinity-labelled and the methionine at position 335 is also labelled. This latter residue is adjacent to lysine 334 which has been shown to be important in higher plant RUBISCOs.

The function of the SSU is unclear. It was first thought that as the Rs. rubrum enzyme has no SSU but has a high Km for CO_2 (the higher plant RUBISCO has a Km for CO_2 that is 10-fold lower), the SSU modulates the affinity of LSU for CO_2 (Christeller & Laing, 1978).

However, L_8S_8 enzymes which have poor affinities for CO_2 do exist (Miziorko & Lorimer, 1983). Another idea was proposed by Gibson & Tabita (1979) who found that in R. sphaeroides, where two forms of the enzyme exist, the L_8 form had a slower rate of activation than did the L_8S_8 form. It was suggested that the SSU may function to increase the activation rate. However, this idea does not hold up to close scrutiny; the LSUs of the two forms of the enzyme do not appear to be identical and so the absence of SSU may not be the only reason for a slower activation (Miziorko & Lorimer, 1983).

More recent work has shown that SSU is essential for catalysis in L_8S_8 carboxylases (Andrews & Ballment, 1983; Gatenby et al., 1985) The first direct demonstration that SSU could influence catalysis was performed by Andrews & Abel (1981). On dissociation of Synechococcus holoenzyme by low pH it was found that loss of even one SSU per molecule could lead to a drop in activity, probably via a conformation change. Subsequent work showed that the catalytic activity of the SSU-depleted enzyme was linearly proportional to the fraction of SSU remaining on the enzyme (Andrews & Ballment, 1983). Neither the $K_m(CO_2)$ nor the carboxylase/oxygenase ratio were affected in these dissociation-reassociation studies. These workers proposed a model in which only LSU with SSU bound is active so that it is LSU-SSU pairs within the RUBISCO holoenzyme that are responsible for activity. This model has been substantiated by the work of Gatenby et al. (1985) who also worked with Synechococcus. The genes for

both LSU and SSU of Synechococcus were cloned into E. coli and it was found that expression of LSU genes alone resulted in no activity, but on expression of SSU genes as well a L_nS_n (where n<8) structure was formed which was enzymically active. This oligomer had a lower specific activity than expected, suggesting that in the assembled RUBISCO all the LSUs may not be complexed with SSU. On increased production of SSU an enhanced enzymic activity was observed.

However, evidence has been presented which suggests that in the prokaryote Chromatium vinosum an L_8 form of RUBISCO exists which is enzymically active (Torres-Ruiz & McFadden, 1985). This finding, if correct, serves only to perpetuate the uncertainty as to the function of the SSUs.

A number of attempts to improve RUBISCO-catalysed carbon-fixation utilising a variety of techniques have been reviewed by Ellis & Gatenby (1984). Those reviewed include attempts to increase the amount of RUBISCO in the cell and attempts to alter the carboxylation/oxygenation ratio. Analysis of RUBISCO from a number of different species has led to the belief that evolution has directed RUBISCO towards a more efficient utilisation of CO_2 and that therefore the balance between oxygenation and carboxylation is not immutable (Jordan & Ogren, 1981). This bodes well for those workers striving towards the goal of increased photosynthetic yield as the result of an alteration of one or both of the subunits of RUBISCO.

Evidence does indeed exist which is consistent with

the view that the carboxylation/oxygenation ratio can be altered in favour of carbon fixation. The substrates of the two RUBISCO-catalysed reactions, O_2 and CO_2, behave competitively towards each another. Oxygen will competitively inhibit the carboxylase reaction with respect to CO_2 and similarly CO_2 will competitively inhibit the oxygenase reaction with respect to O_2 (Laing et al., 1974; Servaites & Ogren, 1978). Thus the relative concentrations of O_2 and CO_2 at the active site of the enzyme are of vital importance in determining whether photosynthetic carbon fixation, and hence plant productivity, is maximal. It has been reported that atmospheric concentrations of CO_2 will not saturate the carboxylase activity of RUBISCO. If the CO_2 concentration of the air is raised 5-fold, then legume crop yield can be increased by between 50% and 100% (Hardy et al., 1978). This increase has been attributed mainly to a decrease in photorespiration, as the raised CO_2/O_2 ratio favours the carboxylation reaction.
This improved competition of the enzyme for CO_2 over O_2 has been achieved naturally in certain species of algae which have been shown to concentrate CO_2 up to 40-fold in relation to the CO_2 concentration present in the external medium (Badger et al., 1980). The so-called C_4 plants similarly achieve an increased CO_2 concentration at the RUBISCO active site by employing a spatial separation of the initial fixation of CO_2 and the subsequent release of this for fixation by RUBISCO (Bowes & Ogren, 1971).

An alternative approach to improving plant productivity is one which involves an alteration of the primary structure of one or both subunits of RUBISCO. Currently, workers are trying to "engineer" a more efficient RUBISCO by initially expressing the genes for LSU and SSU in *E. coli* and allowing the two subunits to come together and assemble. The rationale behind such an approach is to express the genes for both subunits so that a catalytically active enzyme is formed and then to alter these subunits by site-directed mutagenesis, to try to build a better RUBISCO. Expression and assembly of the cyanobacterial RUBISCO in *E. coli* has been achieved (Gatenby *et al.*, 1985). The enzyme synthesised is active and therefore the way ahead is clear for alterations to begin to improve the carboxylase activity.

Site-directed mutagenesis experiments have been initiated for the prokaryote RUBISCO and these have been successful in altering one amino-acid in the LSU of *Rs. rubrum* (Gutteridge *et al.*, 1984). However, the particular mutation performed did not alter the carboxylation/oxygenation ratio and led to a drop in catalysis with an increase in the $Km(CO_2)$.

The higher plant RUBISCOs may well prove more difficult to alter by such *in vitro* site-directed mutagenesis experiments because of the very nature of the mode of synthesis of the subunits, as was described in Section 1.1.3. To date there has been an inability to reconstitute higher plant RUBISCO from isolated subunits *in vitro*. However, the LSU gene of *Z. mays* has been cloned

and expressed in E. coli (Gatenby, 1984). The LSU so formed is insoluble and has no catalytic activity. The next steps in these studies will be to express the SSU gene in the same cell and hope that in the presence of pools of unassembled SSU, holoenzyme will be formed. Such studies are however complicated further by the finding that in isolated chloroplasts, newly-synthesised LSU are bound to another abundant stromal protein (Barraclough & Ellis, 1980), which has been termed the RUBISCO LSU-binding protein (Roy et al., 1982). This finding raises the possibility that a third protein may be involved in the assembly of RUBISCO inside chloroplasts. If this third protein is indeed essential for LSU synthesis and/or its assembly into holoenzyme then it too will have to be present in any in vitro systems which are designed to study RUBISCO assembly. Possible roles of this LSU-binding protein will be discussed more fully in Section 1.3 and Section 4, as it is this protein which was studied in the experiments described in this thesis.

1.2 PHOTOREGULATION OF RUBISCO SYNTHESIS

1.2.1 Photoregulation of gene expression

Higher plants are immobile and as such have acquired a unique set of responses to the environmental stimuli presented to them. One stimulus vital to plant survival is light and a considerable amount of interest has been focussed on

attempting to gain an understanding of the mechanisms by which light can initiate developmental changes during photomorphogenesis. A light stimulus is essential for the development of a photosynthetically-active chloroplast in higher plants; the metabolic pathways associated with photosynthesis and a functional photosynthetic apparatus are present only in plants which have been illuminated (Bradbeer, 1981). A knowledge of light-dependent chloroplast development is therefore important, if the biochemistry of the chloroplast and chloroplast biogenesis are to be understood. If plants are raised in darkness, the proplastids, which develop into chloroplasts in plants which are maintained under continuous illumination or are subjected to a day-night cycle, will develop into etioplasts which lack chlorophyll and a well-organised thylakoid membrane system. On illumination of etiolated plants, the etioplasts develop into chloroplasts. The vast number of biochemical changes which accompany this transition have been reviewed by Bradbeer (1981) and include alterations in the pigment composition and the chloroplast protein spectrum. The protein complements of light-grown and dark-grown plants differ considerably and it has been shown that on illumination of etiolated plants there is a massive increase in the rate of protein synthesis (Lamb & Lawton, 1983). Although some proteins do decrease in abundance upon illumination, most increase.

Among the large number of proteins which accumulate is RUBISCO. In recent years many workers have been striving towards an understanding of the mechanisms by which light

controls gene expression in plants, for ultimately it is altered gene expression which is responsible for many of the changes observed.

There are at least three photoreceptors present in higher plants which are known to mediate the reponses to light. These are phytochrome, protochlorophyllide and a blue-light absorbing photoreceptor. There is evidence that there may be more than one of the latter (Briggs & Iino, 1983). The absorption of light by protochlorophyllide is important in some aspects of chloroplast development which may not be the direct result of altered gene expression (Bradbeer, 1981). Protochlorophyllide is in direct control of chlorophyll synthesis, while the accumulation of the light-harvesting chlorophyll a/b protein (LHCP) is in turn controlled by the availability of chlorophyll. Protochlorophyllide is also thought to be important in thylakoid formation.

Phytochrome is a chromoprotein which is present in plant material in two forms which are distinguishable by monoclonal antibodies (Thomas et al., 1984). In etiolated tissue, phytochrome is present in a form which absorbs red-light (absorption maximum at 655 nm) while after such absorption the chromoprotein is converted into the far-red-light-absorbing form (absorption maximum at 725 nm). It is the latter form of phytochrome which is physiologically active. Illumination of etiolated plants with white light will activate the phytochrome system. Phytochrome is regarded as being responsible for any given response if that response

is elicited by a short illumination with red-light, but then reversed if a far-red-light exposure immediately follows. The properties of, and a large number of the responses mediated by, phytochrome have been reviewed by Smith (1975). The mechanism of action of phytochrome is, however, as yet unknown but it is thought to act by activating or repressing genes differentially (Mohr, 1972). The evidence which has accumulated in favour of this proposal will be reviewed below.

The blue-light photoreceptor(s) is also thought to be important in the control of gene expression. Absorption of ultra-violet light has been shown to induce increases in the rate of synthesis of mRNA for chalcone synthase, an enzyme vital to the flavonoid biosynthetic pathway (Kreuzaler et al., 1983).

There are a number of sites at which light, in principle may cause stimulation or suppression of gene expression. Most of the studies to date on photoregulation in plants have examined chloroplast development and have therefore necessitated an investigation into the affect of light on both nuclear and chloroplast genomes. Light may act by altering the rates of initiation of transcription in either the chloroplast or nucleus, or by affecting the transport of transcripts to the cytoplasm if the genes are nuclear-encoded. Light may also have an effect on the rate of polysome formation by altering the degree to which any particular mRNA is recruited by ribosomes. There may be an alteration in the initiation of translation or in the

transport of nuclear-encoded proteins into the chloroplast. Finally, light may influence post-translational events within the chloroplast stroma or cytoplasm, events which include assembly of multisubunit enzymes and integration of polypeptides into thylakoid membranes. Thus, regulation by light may take place at a number of sites both temporally and spatially.

Post-translational control, effected by light, over the accumulation of one chloroplast protein has been well documented. The light-harvesting chlorophyll a/b protein (LHCP), a chlorophyll binding protein, is not present in etioplasts, although its mRNA is present in etiolated Pisum plants. It is only on illumination of plants that the protein accumulates. Under conditions which prevent chlorophyll accumulation the LHCP apoprotein is degraded (Bennett et al., 1984).

The spectrum of proteins present in plant tissue differs between light-grown and dark-grown plants. These changing patterns of protein synthesis have been shown to be accompanied by changes in the number and size of polysomes (Lamb & Lawton, 1983). The total number of ribosomes appears to increase on illumination of etiolated tissue and there have also been reports that light can mobilise stored mRNA onto polysomes. Similarly, ribosomes have been shown to be more active in the light and peptidyl tRNA concentrations may be increased on illumination (reviwed by Tobin & Silverthorne, 1985).

The above changes may be, in part, responsible for the

altered protein content observed in illuminated tissue. A more specific definition of the level of control has come from findings that the translational activity of certain mRNAs alter on illumination of etiolated plants. In early studies the observed changes in protein concentration were found to correlate with the mRNA activity for a number of proteins. Amounts of translatable mRNA for two nuclear-encoded chloroplast proteins (LHCP and the SSU of RUBISCO) were shown to increase on exposure to light in Lemna (Tobin, 1978). The observed changes in the synthesis of the proteins could be accounted for by changes in their mRNA activities (Tobin, 1981a; Tobin & Suttie, 1980). In Pisum the translatable activity of the mRNA encoding the precursor to the SSU was increased in plants exposed to light (Bedbrook et al., 1980). White light was shown to induce the appearance of mRNA encoding the LHCP apoprotein in Hordeum (Apel & Kloppstech, 1978) and this effect of light was later found to be the result of the action of phytochrome (Apel, 1979).

The work of Bedbrook et al. (1978) first revealed that a chloroplast gene in Zea mays encoding a 32,000Da polypeptide was under photocontrol. The translational activity of the mRNA for this protein was higher in light-grown plants than in dark-grown plants. Similar results were obtained in Sinapis alba by Link (1982), in work in which phytochrome was implicated in the control of the synthesis of this 32,000Da protein, which is thought to be a component of photosystem II (Tobin & Silverthorne, 1985).

An interesting point with regard to the control of mRNA abundance by light is that phytochrome exerts an autoregulatory control over phytochrome mRNA amounts (Colbert et al., 1983). These changes in phytochrome mRNA concentration have been found to occur very rapidly.

The above evidence shows that on illumination the translatable mRNAs for a number of chloroplast proteins increase in concentration, and that in some instances this is phytochrome-controlled. Absorption of light by phytochrome can, however, also lead to a decrease in the activity of particular mRNAs. In Hordeum it has been shown that this photoreceptor will induce a decrease in the translatable mRNA which encodes the enzyme NADPH-protochlorophyllide oxidoreductase, a major membrane protein of the etioplast (Apel, 1981).

Such studies involving determination of translatable mRNA activities for particular proteins do not allow detection of mRNAs which may be sequestered in an untranslatable form. Transcript sequences which require further processing such as polyadenylation or capping will be undetectable. The advent of cloned complementary DNA sequences and their use in hybridisation studies has allowed a more direct and rigorous approach to the determination of amounts of a particular mRNA.

Bollmer & Apel (1983), using a cloned sequence of LHCP as a hybridisation probe, revealed that in Hordeum the steady-state amount of mRNA encoding the LHCP was increased on illumination, and Thompson et al. (1983) showed also by

hybridisation studies that light would cause an increase in the steady-state concentration of SSU mRNA in Pisum. Similarly the work of Stiekema et al. (1983) revealed that in Lemna light could influence the rates of either transcription or degradation of the mRNAs for both SSU and LHCP, as in each case the steady-state level of hybridisable mRNA was found to increase on illumination.

A direct influence of light on transcription has been shown by Gallagher & Ellis (1982). These workers employed cloned DNA probes to examine the abundance of transcripts of LHCP and SSU in the RNA synthesised by nuclei isolated from light-grown and dark-grown Pisum plants. The mRNAs for both proteins were transcribed much more in light-grown plants. Similar results have been found for SSU and LHCP in Lemna, where phytochrome action induces the changes in gene transcription (Silverthorne & Tobin, 1984). Phytochrome action has also been shown to mediate such transcriptional changes in a SSU gene of Glycine max (Berry-Lowe & Meagher, 1985). Further features of the transcriptional control of nuclear genes by light will be described in more detail in Section 1.2.2, where the photocontrol of SSU will be considered more fully.

From the above discussion it is clear that light can affect the expression of both chloroplast and nuclear genes. However, the genes which are affected and the degree to which light controls gene expression do appear to vary among species. In etiolated Hordeum there exists half as much RUBISCO as is present in plants greened for ten hours (Smith

et al., 1974). This is in contrast to the very large dark-light difference in RUBISCO which is seen in Pisum (Smith & Ellis, 1981). Studies using hybridisation probes have also revealed species differences. In etiolated Lemna and Vigna radiata (mung bean) there are substantial amounts of hybridisable SSU mRNA (Stiekema et al., 1983; Thompson et al., 1983) in contrast to the findings with Pisum and Glycine max, where the mRNA for SSU is virtually undetectable in etiolated plants (Bedbrook et al., 1980; Berry-Lowe et al., 1982; Jenkins et al., 1983). In Pisum the LHCP mRNA is, however, readily detectable in dark-grown plants (Jenkins et al., 1983), while in etiolated Hordeum and Lemna, the abundance of LHCP mRNA sequences is low (Gollmer & Apel, 1983; Stiekema et al., 1983). It has been suggested that the differences observed between Pisum and Vigna may be attributable to the extent of chloroplast development at the time of study (Thompson et al., 1983). In dark-grown Vigna, development is arrested at a much later stage and the observed changes on illumination may reflect this. Indeed, Thompson et al. (1983) have found that a number of RNAs are present at high concentrations in dark-grown Vigna but at low concentrations in Pisum. On illumination of plants of the latter species, much more dramatic increases in these RNAs are therefore observed.

There are also reports that the kinetics of the appearance of certain mRNAs in response to light differ in a number of species. In Hordeum the mRNA for LHCP has been

found to increase in abundance within two and a half hours of a red-light treatment (Bollmer & Apel, 1983), while in Pisum very little change is observed in LHCP mRNA abundance for the first 24 hours of illumination (Jenkins et al., 1984). Similar discrepancies arise with the SSU; Jenkins et al. (1984) report no increase in this mRNA until after 24 hours of illumination, while Stiekema et al. (1983) show an increase in SSU mRNA within thirty minutes of red-light illumination in Lemna.

Such kinetic differences may also reflect the stage of development of the chloroplast at the time of study, as discussed above, and/or may be the combined result of a differential effect of light on the various members of the gene families which encode both SSU and LHCP (Berry-Lowe et al., 1982; Dunsmuir & Bedbrook, 1983). It has not yet been determined that the effect of light on all the family members is identical and it is possible that while some genes are enhanced in their expression by light, others may be expressed constitutively and some may be inactive pseudogenes. It has been reported that one of the SSU genes of Pisum is selectively expressed at different stages in development and in different tissues, thus suggesting differential transcriptional control (Coruzzi et al., 1984). Further information on the control of expression of the individual genes of a gene family awaits the development and use of specific gene probes. Such probes have been reported to have been produced for six of the SSU genes of Lemna (Tobin & Silverthorne, 1985) and some of the

preliminary findings of this work will be discussed in Section 1.2.2 below, in a consideration of the photoregulation of SSU.

1.2.2 Photoregulation of RUBISCO synthesis

The effect of light on the biosynthesis of RUBISCO has long been a subject of interest. It has been demonstrated that light, acting through phytochrome, is responsible for an increase in RUBISCO activity in Pisum (Graham et al., 1968). Concomitant with this increase in activity there is an increase in RUBISCO protein. A red-light treatment has also been found to increase the activity of RUBISCO in Zea mays. and this increase can be inhibited by treatment with cycloheximide (Kobayashi et al., 1980). A similar inhibition of RUBISCO accumulation by antibiotics is seen in plants of Hordeum subjected to the light (Kleinkopf et al., 1970). Thus, early work suggested that light treatment resulted in an increase in enzyme protein. I intend to discuss the photoregulation of the biosynthesis of both subunits of RUBISCO separately, beginning with the effect of light on the SSU. After a consideration of the photocontrol of LSU, the degree to which light can coordinate the synthesis of both subunits will be examined.

The first report that illumination by white light led to increases in SSU protein, resulting from increased mRNA amounts, appeared in 1978 in studies with Lemna (Tobin, 1978). Tobin & Suttie (1980) later showed that the observed

increases in the mRNA amount were sufficient and rapid enough to account for the increase in SSU protein and Tobin (1981b) demonstrated that phytochrome was the photoreceptor responsible. Immunodetection studies of the proteins of greening Pisum plants have revealed that SSU is not present until the plants have received 24 hours of white light (Jenkins et al., 1983). This increase in SSU protein has been shown to be closely correlated with the increases in translatable SSU mRNA (Sasaki et al., 1981; Bennett et al., 1984) and it has been demonstrated that phytochrome controls this induction in Pisum (Sasaki et al., 1983; Jenkins et al., 1983).

The use of cloned hybridisation probes by Smith & Ellis (1981) revealed that in Pisum the transcripts for SSU were more abundant in light-grown than in dark-grown plants and concluded that photocontrol of SSU biosynthesis was therefore at the level of RNA synthesis or breakdown. A careful examination of the cellular location of the SSU gene transcripts by the same workers revealed that light did not simply cause a transport of stored SSU transcripts to the cytoplasm for translation. Stiekema et al. (1983) reached a similar conclusion in a study of SSU synthesis in Lemna. Hybridisation studies confirmed that the increases in SSU protein and mRNA occurred in parallel. In Pisum, SSU mRNA is undetectable in etiolated seedlings but amounts slowly rise over the first 24 hours of illumination; the maximum amount of mRNA is detectable 36 hours after illumination (Jenkins et al., 1984). This correlates with the appearance

of SSU protein after 24 hours of illumination, as detected by immunological techniques (Jenkins et al., 1983).

As discussed above in Section 1.2.1, the control of SSU synthesis has been shown to occur at the level of transcription. Gallagher & Ellis (1982) were the first to demonstrate that an increase in transcription of SSU genes was responsible for the observed increase in SSU protein upon illumination. The transcription rate in light-grown plants was found to be 18-fold greater than in plants grown in darkness. More recently Berry-Lowe & Meagher (1985) have found that there is a 16 to 32-fold increase in the transcription of one SSU gene on illumination of Glycine max plants and that this increase is controlled by phytochrome.

The transcriptional control of SSU gene expression in Glycine max does, however, differ in some respects from that which is observed in Pisum. In Pisum a number of rapid changes in the transcription rate of SSU are observed under certain conditions (Gallagher et al., 1985). The first of these is a transient increase in transcription on transfer of etiolated plants to the light. This transient increase occurs within the first hour of illumination, but within the following 4-5 hours of illumination there is a drop in transcription. Following this drop is a slow, steady rise in transcription over the next 20 hours and a maximum rate is reached after 36 hours of illumination of etiolated plants. Rapid changes are also observed when greened Pisum plants are returned to darkness for five hours. After such a period the transcription rate of SSU genes declines by 75%.

The authors concluded that as the dark-reversion of phytochrome to its physiologically inactive form is a slow process (Smith, 1975), some other type of photoregulation may be operating. In contrast, when light-grown Glycine max plants are placed in darkness there is a slow reduction in SSU gene transcription which continues over a 48 hour period and this is thought to be the result of the dark reversion of phytochrome (Berry-Lowe & Meagher, 1985).

The degree of control by light over individual members of the SSU gene family has yet to be investigated. It has been reported that six gene-specific probes have been developed for the SSUs of Lemna and red-light illumination will result in an increase in the amounts of RNA for each of these (Tobin & Silverthorne, 1985). As differential control of transcription of the SSU genes has been reported (Coruzzi et al., 1984), differences in the expression of individual SSU genes, as controlled by light, may be expected.

The application of recombinant DNA technology has also helped to gain an insight into the molecular mechanisms of photoregulation. Broglie et al. (1984) have demonstrated that on transfer of one member of the SSU gene family to Petunia callus cells via a Ti plasmid vector, there is expression of the SSU gene and this expression occurs in a light-regulated manner, similar to that which is observed in Pisum. Similarly, Schreier et al. (1985) have used the promoter and transit peptide sequences of the SSU gene to bring about transport of a foreign gene (neomycin phosphotransferase II) into Nicotiana tissue, and have

found that expression of this gene in Nicotiana chloroplasts is light-regulated.

The use of such in vivo expression systems will permit examination of the effect of various mutations within the SSU genes and so allow the DNA regions essential for light-regulation to be defined. Morelli et al. (1985) have demonstrated that a 33 base pair sequence close to the TATA box sequence of one SSU gene is all that is required to confer light-inducibility on that particular SSU gene. The sequence contains a ten base pair length which is conserved in all of the SSU genes so far sequenced from a number of species.

Siddell & Ellis (1975) have shown that etioplasts from Pisum will synthesise RUBISCO LSU if ATP is supplied as an energy source. Some LSU appears to be assembled into holoenzyme in dark-grown plants since very low amounts of RUBISCO are detectable when extracts of etiolated Pisum plants are subjected to electrophoresis on non-denaturing polyacrylamide gels (Smith & Ellis, 1981). Immunoblotting techniques have allowed detection of LSU in Pisum plants which have been greened for six hours (Jenkins et al., 1983).

Hybridisation studies have revealed the presence of LSU mRNA in dark-grown Pisum plants, in contrast to the situation with SSU (Shinozaki et al., 1982; Jenkins et al., 1984). Thus the RUBISCO LSU is not photoregulated in the same manner as the SSU and it may be that the photocontrols on both subunits differ. A further contrast

between the photoregulation of LSU and SSU is seen in the lack of a close correlation between the increase in LSU protein and LSU mRNA on illumination of etiolated plants. There is a large increase in LSU mRNA over the first 12 hours and although the LSU protein is detectable after six hours, it only begins to accumulate after 24 hours of illumination (Jenkins et al., 1984). The accumulation of the LSU therefore lags behind that of its mRNA and so control of LSU gene expression appears not to be solely at the mRNA level. Post-translational controls may operate.

There are also a number of conflicting reports with regard to the control of LSU gene expression by phytochrome. Sasaki et al. (1983) using five-day old Pisum plants reported phytochrome control of the amount of LSU mRNA and Thompson et al. (1983) also showed that this photoreceptor could evoke responses in LSU mRNA amounts in six to eight-day old Pisum plants. In contrast, Jenkins et al. (1983) have found that both far-red and red-light will induce an increase in LSU protein in eight-day old Pisum plants, while Link (1982) using cloned fragments of DNA which encoded the LSU, as probes for LSU mRNA, found no phytochrome control over LSU gene expression in Sinapis alba. Jenkins et al. (1983) have stated that as the Pisum plants used by them were somewhat older than those employed by other workers, the ability of the plant to respond to phytochrome may be a function of the stage of chloroplast development.

In Vigna radiata there is less light-dark inducibility of LSU than is observed in Pisum (Thompson

et al., 1983). These workers also showed that the plastid amount of DNA differed between the two species, with amounts being higher in dark-grown Vigna. This too suggests that the state of development of the chloroplast has an important effect on the control of gene expression.

Finally evidence pertinent to the question of coordinated synthesis of the RUBISCO subunits will be considered. As has been discussed above, the photocontrols on the two subunits differ (Jenkins et al., 1983). The expression of LSU and SSU genes can be dissociated from one another by treating etiolated Pisum seedlings with far-red light. In this case LSU is detectable after six hours illumination of etiolated plants, whereas SSU is only detectable after 24 hours illumination (Jenkins et al., 1983). Similar findings have resulted from studies with Zea mays; in this species there is an observable increase in LSU protein, as detected by antibody, prior to the increase in SSU (Nelson et al., 1984). Conversely, in another C_4 plant, pearl millet (Panicum americanum), it has been found that there is an excess of SSU over LSU in etiolated plants (Bassett et al., 1985).

However, some findings do suggest a coordinated regulation of subunit synthesis. In studies with Pisum plants Sasaki et al. (1981) have shown that a burst in SSU protein synthesis is observed after 24 hours of illumination of etiolated plants and that this coincides with a large increase in RUBISCO accumulation. The accumulation of RUBISCO parallels the increase in SSU and so suggests a control of

LSU induction and assembly in some manner by SSU. This large increase in SSU synthesis after 24 hours of light treatment was also seen by Jenkins et al. (1983), who found that the LSU, although previously low in concentration, began to increase as SSU protein began to accumulate.

1.3 THE RUBISCO LSU-BINDING PROTEIN

1.3.1 Historical background

It has been demonstrated that RUBISCO assembly will proceed in isolated chloroplasts (Chua & Schmidt, 1978; Smith & Ellis, 1979; Barraclough & Ellis, 1980) but little is known with regard to the mechanism by which the LSUs and SSUs interact inside the chloroplast to yield holoenzyme. However, information concerning some of the post-translational events related to the synthesis of both subunits prior to their incorporation into RUBISCO has been published and this work will be presented below.

As described in Section 1.1.3 above, the SSU is synthesised on cytoplasmic ribosomes as a precursor and this is imported into the chloroplast where processing and assembly proceed. It has been shown that 80% of the Pisum SSU precursor, synthesised in a wheat-germ cell-free translation system from poly A-containing mRNA, is imported into isolated chloroplasts and therein correctly assembled into holoenzyme (Chua & Schmidt, 1978). This finding reveals that endogenous LSUs are competent to combine with imported

SSUs and so yield RUBISCO. The work of Smith & Ellis (1979) showed unequivocally that incorporation of SSU into holoenzyme occured within the stromal compartment of the chloroplast.

The events involved in the assembly of LSU into RUBISCO appear to be complex. Some early work revealed that LSUs synthesised in isolated Spinacia chloroplasts were able to assemble into RUBISCO holoenzyme (Bottomley et al., 1974), while other workers reported a failure of isolated chloroplasts to incorporate newly-synthesised LSU into holoenzyme (Blair & Ellis, 1973). Both of these findings resulted from analyses of chloroplast proteins on polyacrylamide gels run under non-denaturing conditions. Careful consideration (Ellis, 1977) of the analytical procedures employed in such studies revealed that the apparent assembly of LSU noted by Bottomley et al. (1974) was due to fortuitous comigration of newly-synthesised, but unassembled, LSU with pre-existing RUBISCO on non-denaturing polyacrylamide gels. These unassembled LSUs were subsequently found to migrate as a complex which had a higher molecular weight than that of holoenzyme (Ellis, 1977).

Later work further investigated the finding that newly-synthesised RUBISCO LSU was not immediately incorporated into holoenzyme; it was the work of Barraclough & Ellis (1980) which first revealed the possible involvement of a third protein in the assembly of the holoenzyme. These workers demonstrated a lag in the assembly of RUBISCO. This was determined in a non-denaturing polyacrylamide gel

analysis of the incorporation of radiolabelled LSU into holoenzyme, in which proteins isolated from chloroplasts incubated with radiolabelled methionine under conditions suitable for protein synthesis were subjected to electrophoresis. It was found that newly-synthesised LSU did not assemble into holoenzyme in isolated chloroplasts until after more than 30 minutes incubation. For the first 30 minutes of the incubation period the newly-synthesised LSUs were present in a band which migrated more slowly than RUBISCO on 5% non-denaturing polyacrylamide gels. Subsequent electrophoresis of this slowly-migrating band under denaturing conditions revealed that the newly-synthesised, but unassembled, LSUs were non-covalently associated with a chloroplast protein of approximate subunit molecular weight 60,000. This latter protein was not radiolabelled when isolated chloroplasts were incubated with radioactive amino-acids under conditions suitable for protein synthesis. This chloroplast protein, of subunit molecular weight 60,000, which is associated with newly-synthesised LSU has been termed the LSU-binding protein, while the slowly-migrating species detectable on non-denaturing gels is termed the LSU-binding protein complex (Roy et al., 1982).

The interesting possibility that the LSU-binding protein complex may be a precursor in the assembly of RUBISCO was raised by the further finding of Barraclough & Ellis (1980) that, when isolated chloroplasts are incubated and illuminated in the presence of radiolabelled methionine, and products of chloroplast protein synthesis examined by

non-denaturing polyacrylamide gel electrophoresis, the radiolabelled LSU migrated initially with the LSU-binding protein complex, but after 30 minutes of incubation radiolabelled LSU was present within the holoenzyme. This suggested that the newly-synthesised LSU was moving into RUBISCO via the LSU-binding protein complex. However, the non-denaturing gel analysis does not rule out the possibility that other lower molecular weight forms of unassembled, newly-synthesised LSU are present in isolated chloroplasts which are competent to assemble into holoenzyme. Barraclough & Ellis (1980) were careful to point out that the non-denaturing polyacrylamide gel analysis did not provide unambiguous evidence for a precursor-product relationship between the LSU-binding protein complex and RUBISCO because there was much insoluble radioactivity present at the top of the gel, which may have included unassembled LSU. Indeed, the presence of such unassembled LSU has been demonstrated (J.E. Musgrove & R.J. Ellis, pers. comm.).

Since the initial discovery by Barraclough & Ellis (1980) that newly-synthesised LSU is not immediately assembled into holoenzyme but is associated with the LSU-binding protein, much work has centred on determining the properties of the latter, the long term goals being an elucidation of the function of the LSU-binding protein and the mechanism of assembly of RUBISCO.

The LSU-binding protein subunit has been shown to have a peptide map which differs from that of LSU (Bloom et al., 1983). Antibodies raised against the LSU-binding protein have

been used to demonstrate that the protein is nuclear-encoded and careful examination of the LSU-binding protein by SDS polyacrylamide gel electrophoresis has revealed that two subunit types of molecular weights 60,000 and 61,000 exist (Hemmingsen & Ellis, 1986). Antibodies raised against the LSU-binding protein have been used in immunoblotting studies and these have revealed the presence of the protein in chloroplasts from Triticum, Hordeum and Pisum and also in leucoplasts prepared from the endosperm of Ricinus communis seeds (Hemmingsen & Ellis, 1986). The latter tissue is known to possess much RUBISCO, albeit of unknown function.

The LSU-binding protein complex can be dissociated by ATP into its constituent LSU-binding protein subunits and associated newly-synthesised LSU (Bloom et al., 1983). No SSU can be detected in the LSU-binding protein complex (Barraclough & Ellis, 1980). Dissociation by ATP is dependent upon the presence of magnesium ions and does not occur in the presence of non-hydrolysable analogues of ATP (Bloom et al., 1983). However, these authors did not demonstrate that ATP hydrolysis occurred in the dissociation reaction; indeed Hemmingsen & Ellis (1986) were unable to show that the LSU-binding protein is either adenylated or phosphorylated in the reaction and stated that it may therefore be the differing stereochemistry of the analogue which prevents dissociation of the LSU-binding protein complex.

1.3.2 Possible roles of the LSU-binding protein

A number of possible roles for the LSU-binding protein have been proposed since its discovery. In this section the evidence in favour of each of these will be presented. The work of Roy *et al.* (1982) confirmed the initial findings of Barraclough & Ellis (1980) that, in isolated chloroplasts, newly-synthesised LSU was associated with another chloroplast protein. Their work extended the earlier findings by showing, in a pulse-chase study, using isolated chloroplasts incubated with radiolabelled amino-acids, that the radiolabelled LSU entering the holoenzyme during the chase period had been synthesised earlier, and was not the result of continued chloroplast protein synthesis.

Such results from studies of LSU incorporation into RUBISCO suggest that pools of unassembled LSU are present within chloroplasts. Pools of unassembled LSU and SSU have been demonstrated in *Pisum* leaves (Roy *et al.*, 1978b; Roy *et al.*, 1979) and in chloroplasts isolated from plants labelled *in vivo* (Roy *et al.*, 1982). The unassembled SSU appears to be present as monomers in chloroplasts (Roy *et al.*, 1979). The unassembled LSUs detected by Roy *et al.* (1979; 1982) were present in two different forms in the chloroplast. The first of these forms sedimented faster in sucrose density gradients than does RUBISCO and these LSUs are associated with the LSU-binding protein. The second form of LSU observed by Roy and his coworkers had a sedimentation coefficient of 7s. Determination of the molecular weight of

these LSUs suggest that, in this form, the LSU is present as a dimer or as a heterodimer, with the second protein having a subunit molecular weight of around 60,000 (Roy et al., 1982). This second form of LSU was not present on the non-denaturing gels examined by Barraclough & Ellis (1980)

It has been suggested that the LSUs which assemble into RUBISCO on incubation of isolated chloroplasts are those which have come from either, or both, of these two pools of unassembled LSU (Roy et al., 1982). Milos & Roy (1984) have presented evidence which suggests that the LSU-binding protein complex does release LSUs which are available for assembly into holoenzyme. Incubation of a chloroplast lysate with MgATP was found to release LSUs from the complex, with an accompanying increase in the LSU content of the pool which sediments in a sucrose density gradient at 7s. Further incubation of the lysate resulted in incorporation of LSUs into holoenzyme. The authors stated that there were insufficient LSUs present in the LSU pool of sedimentation coefficient 7s to account for the observed assembly. It was therefore concluded that the LSU-binding protein complex was important in RUBISCO assembly, having a role either as an intermediate in the assembly reaction or as a storage site for unassembled LSUs which can be released by ATP when there are sufficient SSUs available.

It was Ellis et al., (1980) who first proposed the idea that the LSU-binding protein may function to maintain the solubility of LSUs prior to the incorporation of these into RUBISCO. This, as a role for the LSU-binding

protein, is in accord with the known physical properties of the LSU. The higher plant LSU, when isolated from the holoenzyme, is relatively insoluble in aqueous media (see Section 1.1.2). The work of Gatenby (1984) has shown that expression of cloned genes for the LSU of Zea mays in E. coli leads to production of an insoluble LSU, suggesting that this insolubility is a problem intrinsic to LSU. Thus a further chloroplast component may be necessary to maintain LSU solubility prior to its assembly. It may be that this component is SSU; Gatenby (1984) suggests that LSU-SSU heterodimers may form which would then self-assemble inside the chloroplast, although to date no such dimers have been detected.

The LSU-binding protein has so far not been identified with any other known chloroplast protein, hence leaving the question of an unambiguous function unanswered. This does not, however, obviate the need to examine all properties of the protein until such time as a function can be elucidated.

1.4 AIMS OF THE PROJECT

The aims of this project were essentially two-fold. The first of these aims was to confirm the existing information concerning the RUBISCO LSU-binding protein, in particular the finding that a relatively abundant chloroplast protein of molecular weight 60,000 is associated with newly-synthesised, unassembled LSU. Secondly, the project was designed to extend our knowledge of this potentially

important protein in two types of study. Initially, antibodies raised against the LSU-binding protein were to be used to investigate certain developmental aspects of the LSU-binding protein. These were to include a study of the effect of light on the synthesis of the LSU-binding protein and to determine if there existed any correlation between the accumulation of the LSU-binding protein and either of the subunits of RUBISCO. Secondly, the ATP-dependent dissociation of the LSU-binding protein complex was to be investigated, to determine the fates of both the LSU-binding protein and the associated LSUs, with a view to proposing a physiological role for this dissociation.

SECTION TWO - MATERIALS AND METHODS

2.1 GROWTH OF PLANTS

Seeds of Pisum sativum (var. Feltham First) were obtained from Charles Sharpe and Co. Ltd., Sleaford. Seeds were sown in compost (Fisons Levington compost, John Astley and Son Ltd.) and grown under warm white fluorescent lights (Phillips) with a photon fluence rate of 50 μmoles m^{-2} s^{-1}. The photoperiod was 12 hours. The growth room temperature was maintained at 20°C ± 2°C and peas watered as necessary. The age of plants was measured from time of sowing.

Dark-grown peas were cultivated as described above except these were maintained in total darkness at 20°C for periods of 8 to 10 days from the time of sowing. After 8 days some plants were transferred to a controlled environment cabinet at 20°C and maintained under continuous white light for up to 48 hours. Illumination was provided by warm white fluorescent tubes (photon fluence rate of 200 μmoles m^{-2} s^{-1}).

At harvest dark-grown and greened pea apices were excised, weighed and frozen by dropping them directly into liquid nitrogen. Apices were stored at -80°C until required.

2.2 EXTRACTION OF TOTAL SOLUBLE PROTEINS FROM PEA APICES

Soluble protein was extracted by the method of Smith & Ellis (1981). Dark-grown or greened apices, stored at -80°C, were thawed and placed in a mortar at 0°C. Ten

apices were ground in 10 ml of the following buffer:

50 mM Tris-HCl
1 mM EDTA
2 mM PMSF
10 mM 2-mercaptoethanol.
pH = 8.0 (at 20°C)

Homogenisation was continued until a uniform slurry was obtained. The homogenate was centrifuged at 18,000 x g for 20 minutes at 4°C. The supernatant was removed and made up to a volume of 10 ml by the addition of further extraction buffer which gave a soluble protein extract equivalent to one apex per millilitre. This extract was divided into two portions, one of which was stored at 4°C (non-denatured extract) while SDS was added to the remainder to give a final concentration of 2% (w/v).The latter extract was boiled for 5 minutes and stored at -20°C until required (denatured extract). The pellet containing membranes was retained for the assay of chlorophyll (see Section 2.7.2).

2.3 CHLOROPLAST ISOLATION

2.3.1 Isolation technique

Chloroplasts were isolated according to the method of Blair & Ellis (1973) from 10-day old pea plants grown under a 12 hour photoperiod. Pea leaves (25 g) in 100 ml of

sterile ice-cold sucrose isolation medium (SIM) were homogenised in a Polytron (Northern Media Supplies, Hull) at setting 10 for 8 seconds. The composition of SIM was:

0.35 M sucrose
25 mM HEPES-NaOH
2 mM EDTA
2 mM sodium isoascorbate
pH = 7.6

The sodium isoascorbate was added just before use. The homogenate was filtered quickly through 8 layers of muslin and centrifuged at 3,200 x g and 4°C for one minute. The supernatant was discarded and the pellet resuspended in a small volume of cold SIM and recentrifuged. This pellet constitutes "washed" chloroplasts.

2.3.2 Radiolabelling of chloroplast proteins

Washed chloroplasts (Section 2.3.1) were resuspended in 1 ml of sorbitol resuspension medium which had the following composition:

0.33 M sorbitol
50 mM Tricine-KOH
pH = 8.4

To this was added 250 µCi of [^{35}S]-methionine (1000 Ci

mmole^{-1}). Chloroplasts were incubated according to the method of Blair & Ellis (1973) at 20°C in an illuminated water bath for up to 30 minutes. The light intensity was 100 µmoles m^{-2} s^{-1}. Aliquots removed from the incubation were centrifuged at 10,000 x g at 4°C in an Eppendorf 5412 microfuge for 10 minutes. The supernatant was discarded and the pellet resuspended in a small volume of 10 mM Tris-HCl (pH 7.6) to lyse the chloroplasts. The lysate was recentrifuged and the supernatant retained. This supernatant contained labelled stromal proteins synthesised by chloroplast ribosomes.

The degree of ^{35}S-methionine incorporation into protein was estimated by determination of acid-insoluble counts as described by Siddell & Ellis (1975). Aliquots (5 µl) from the chloroplast incubation were pipetted onto Whatman No. 1 filter paper squares of dimensions 1 cm x 1 cm and allowed to air dry. The filter paper pieces were then placed in trichloracetic acid (20% (v/v)) which was heated to 100°C. The filter paper pieces were left in the hot trichloroacetic acid (TCA) for 20 minutes after which time the TCA was replaced with fresh and the filters washed. The filters were then washed twice with ethanol and once with ether before being dried under a stream of nitrogen. The dried filters were placed in scintillation vials with Beckman NA scintillation fluid (4 ml) and the vials were counted in a LKB Minibeta 1212 scintillation counter.

2.3.3 Synthesis of chloroplast proteins in chloroplast stromal extracts

Chloroplasts were isolated as described in Section 2.3.1. The chloroplasts were lysed by resuspension in a buffer containing the following:

25 mM Tris-HCl
10 mM $MgSO_4$
pH = 8.0

The lysate was centrifuged in an Eppendorf 5412 microfuge for 10 minutes at 10,000 x g and 0°C to remove membranes. ATP, GTP and KCl were added to the supernatant from a stock solution to give final concentrations of 2 mM, 0.2 mM and 80 mM respectively. This supernatant was incubated at 25°C to allow protein synthesis to proceed (Ellis & Hartley, 1982). After incubation the stromal proteins were analysed by electrophoresis on polyacrylamide gels (see Section 2.6).

2.4 PROTEIN PURIFICATION

The method followed was that developed by Hemmingsen & Ellis (1986). In this protocol RUBISCO and the RUBISCO LSU-binding protein complex are initially co-purified by gel filtration and then separated from each another by ion-exchange chromatography on DEAE-Sephacel.

Leaves (600 g) from 10 to 12-day old pea plants were

ground in SIM at 4°C. Batches of leaves weighing approximately 80 g were homogenised in 200 ml volumes of SIM using a Polytron at setting 10 for 8 seconds. Homogenates were filtered through 8 layers of muslin and the filtrates centrifuged at 3,200 x g and 4°C for 1 minute. The supernatants were discarded, the chloroplast pellets resuspended in 3 ml volumes of 10 mM Tris-HCl (pH 8.0) and pooled. The suspension was incubated on ice with occasional gentle agitation for 20 minutes to ensure complete chloroplast lysis. Membranes were removed by centrifugation at 10,000 x g and 4°C for 20 minutes. The supernatant (chloroplast stroma; approximately 50 ml) was retained, maintained at 4°C and sufficient solid ammonium sulphate added over a period of 30 minutes to bring the supernatant to 40% saturation (22.6 g per 100 ml). The precipitated protein was removed by centrifugation at 10,000 x g and 4°C for 20 minutes and the supernatant retained. Sufficient solid ammonium sulphate was added to the supernatant to bring this to 70% saturation (18.7 g per 100 ml). The precipitated protein was collected by centrifugation at 10,000 x g and 4°C for 20 minutes. The pellet was resuspended in 2 ml of 10 mM Tris-HCl (pH 7.6) and solid sucrose added to give a final concentration of 5% (w/v). The protein was loaded onto a Sephacryl S300 superfine (Pharmacia) column (diameter 3 cm; height 90 cm) equilibrated in 20 mM Tris-HCl (pH 7.6). The buffer was pumped through the column at 15 ml h^{-1} using a LKB Microperpex peristaltic pump and fractions (7 ml) collected in chromic acid-washed test tubes at 4°C by means of a LKB Redirac

fraction collector. For all fractions collected the absorbance at 280 nm was determined and from the resulting elution profile the position of RUBISCO was estimated. Fractions were stored at 4°C while aliquots were run on a 5% non-denaturing gel (see Section 2.6.2) to determine which fractions contained the RUBISCO LSU-binding protein complex. Fractions containing the peak of the RUBISCO LSU-binding protein complex eluted 2 or 3 fractions ahead of the peak RUBISCO-containing fractions and it was therefore possible to separate and pool the peak fractions corresponding to each.

The second step in the purification procedure involved ion exchange chromatography on a DEAE-Sephacel (Pharmacia) column (diameter 4.5 cm; height 90 cm). The column was operated under gravity at a flow rate of 8 ml min^{-1}. The RUBISCO and RUBISCO LSU-binding protein complex from the gel filtration column were loaded onto this column separately. The column was equilibrated in 100 mM KCl dissolved in 10 mM Tris-HCl (pH 7.6) prior to the loading of the pooled RUBISCO fractions. After loading, the column was washed extensively in the above buffer before the RUBISCO was eluted in 200 mM KCl dissolved in 10 mM Tris-HCl (pH 7.6). Fractions (8 ml) were collected in chromic acid-washed tubes using a Redirac fraction collector. The absorbance at 280 nm was determined and those fractions containing protein were pooled and stored at 4°C. The column was washed with 300 mM KCl dissolved in 10 mM Tris-HCl (pH 7.6) to remove the small amount of RUBISCO LSU-binding protein complex which eluted with the RUBISCO from the gel filtration column and these

fractions were added to the RUBISCO LSU-binding protein complex fractions obtained from the gel filtration column. The DEAE-Sephacel column was washed by the passage of 2 column volumes of 2 M KCl dissolved in 10 mM Tris-HCl (pH 7.6) to remove all remaining bound proteins and equilibrated with 200 mM KCl dissolved in 10 mM Tris-HCl (pH 7.6). The pooled RUBISCO LSU-binding protein complex fractions were loaded and contaminating RUBISCO removed by washing with 2 column volumes of 200 mM KCl dissolved in 10 mM Tris-HCl (pH 7.6). The complex was eluted with 300 mM KCl dissolved in 10 mM Tris-HCl (pH 7.6) as described above.

At this stage the purity and identity of the RUBISCO and the complex were determined by SDS gel electrophoresis (Section 2.6.1). If impure, the RUBISCO fraction was further purified by passage through the DEAE-Sephacel column as descibed above. The RUBISCO LSU-binding protein complex was further purified and concentrated by passage through a DEAE-Sephacel column (8 ml bed volume) equilibrated in 200 mM KCl dissolved in 10 mM Tris-HCl (pH 7.6). The fraction was loaded onto the column and the column extensively washed with the equilibration buffer. The complex was eluted with 300mM KCl dissolved in 10 mM Tris-HCl (pH 7.6) and fractions collected manually in Eppendorf tubes. The fractions containing protein were pooled and purity again determined.

Sufficient glycerol was added to the purified RUBISCO fraction to give a 20% (v/v) solution. This and the untreated RUBISCO LSU-binding protein complex fraction were stored at -20°C.

2.5 IMMUNOLOGICAL TECHNIQUES

2.5.1 Production and purification of antibodies

RUBISCO and the RUBISCO LSU-binding protein complex were purified as described above (Section 2.4) and used in the immunisation of New Zealand White rabbits. Rabbits were injected intramuscularly. Between 100 µg and 200 µg of RUBISCO and between 40 µg and 180 µg of the RUBISCO large subunit-binding protein complex were injected each time. In the first injection the purified proteins were mixed with an equal volume of Freund's complete adjuvant (Grand Island Biological Co. Ltd.) while subsequent injections contained incomplete adjuvant. Each rabbit received 6 booster injections at monthly intervals. Blood was collected from the ear vein and allowed to clot at room temperature, serum was removed by pipette and centrifuged at 3,000 x g for 30 minutes at 4°C to remove red blood cells. This yielded antiserum used throughout the course of this work. Antiserum raised against RUBISCO was stored at -20°C with no further treatment.

Crude antiserum raised against the RUBISCO LSU-binding protein complex was purified by affinity chromatography following the method of Mains & Eipper (1976) in which purified RUBISCO was covalently linked to cyanogen bromide-activated Sepharose 4B (Pharmacia). Sufficient solid ammonium sulphate was added to a solution of purified RUBISCO

(22 mg dissolved in approximately 10 ml of 10 mM Tris-HCl, pH 7.6) to give 70% saturation (43.5 g per 100 ml). Precipitated protein was recovered by centrifugation at 18,000 x g and 4°C for 20 minutes. The supernatant was discarded and the pellet resuspended in 2 ml of coupling buffer:

0.1 M $NaHCO_3$

0.5 M NaCl

pH = 8.3

The protein was then loaded onto a Pharmacia PD 10 desalting column (Sephadex G25 medium) which had been equilibrated in coupling buffer. The column was eluted with coupling buffer. All fractions containing RUBISCO were retained (volume = 6 ml) at 4°C until required. The cyanogen bromide-activated Sepharose (1.5 g) was swollen on a sintered glass filter by the passage under vacuum of 300 ml of 1 mM HCl. The Sepharose was then washed with 7.5 ml of coupling buffer, excess buffer removed by suction and the Sepharose washed into the RUBISCO solution with 4 ml of coupling buffer. This gave a gel:buffer ratio of 1:2. All subsequent steps were carried out at 4°C. The protein was coupled to the sepharose overnight with continuous rotation. The gel was allowed to settle and coupling buffer was removed by pipette. Blocking buffer (10 ml) was added:

0.1 M $NaHCO_3$

0.2 M glycine

pH = 8.0

The Sepharose-protein conjugate was incubated overnight in blocking buffer with continuous rotation. The Sepharose was washed in coupling buffer and poured into a 5 ml syringe. The column was washed once in sodium acetate buffer (pH 4.0) and once in coupling buffer before use. The affinity resin contained 4 mg of RUBISCO per millilitre of resin.

Crude antiserum (5 ml) was diluted threefold with 50 mM Tris-HCl (pH 7.6) and was applied to the column at 4°C at a flow rate of 8 ml h^{-1}. The column was eluted with 50 mM Tris-HCl (pH 7.6) and fractions were collected immediately. The filtered serum was recovered and stored in aliquots at -20°C. This constituted "clean" antiserum to the RUBISCO LSU-binding protein complex.

After use the column was washed with the following buffer to remove bound anti-RUBISCO antiserum:

8 M urea

0.1 M $NaHCO_3$

pH = 8.3

The column was stored in coupling buffer containing 2% (w/v) sodium azide at 4°C.

2.5.2 Preparation of iodinated protein A

A 1 mg ml^{-1} stock solution of *Staphylococcus aureus* protein A was prepared by dissolving the protein in a phosphate-buffered saline solution (PBS):

138 mM NaCl

2.82 mM KCl

7.37 mM Na_2HPO_4

1.46 mM KH_2PO_4

pH = 7.4

Protein A solution (25 µl) was then added to 1 mCi of $Na^{125}I$ (Amersham; 13.5 mCi μg^{-1}) with 10 µl of a stock solution of chloramine T (2 mg ml^{-1}) freshly prepared in potassium phosphate buffer (pH 7.5). These solutions were mixed well and incubated at room temperature for 2 minutes. After this time 25 µl of a tyrosine solution (2 mg ml^{-1}), 50 µl of 10% (w/v) bovine serum albumin (BSA) and 200 µl of PBS were added. The tyrosine solution and BSA were prepared in potassium phosphate buffer (pH 7.5). The iodinated protein A was separated from free iodine by gel filtration in a 5 ml column (bed height = 20 cm) containing Sephadex G50 medium grade (Pharmacia). The column was prewashed with 10 ml of 10% (w/v) BSA dissolved in PBS and then eluted with PBS. Fractions (200 µl) were collected, aliquots counted in an LKB 1280 Ultrogamma counter and those containing iodinated protein stored at 4°C.

2.5.3 Analysis of proteins by Immunoblotting ("Western" blotting)

Samples to be examined were subjected to polyacrylamide gel electrophoresis under denaturing conditions as described in Section 2.6. After electrophoresis the stacking gel was cut away with a scalpel and the remainder of the gel trimmed if necessary. The gel was then washed in "Western" transfer buffer of the following composition:

192 mM glycine
25 mM Tris base
20% (v/v) methanol

The gel was placed on a "Scotch-brite" pad and a piece of nitrocellulose (Schleicher and Schull; pore size 0.45 μm) cut to fit the gel exactly. The nitrocellulose was soaked in "Western" transfer buffer and laid over the gel, care being taken to avoid bubbles between layers. A second "Scotch-brite" pad was placed over the nitrocellulose.

Transfer was performed as described by Vaessen et al. (1981). The gel sandwich was placed in a Trans-Blot cell (Biorad) which contained 3 litres of transfer buffer. Transfer was performed at room temperature for 2 hours at 60 volts. After transfer, filters were incubated with agitation for 1 hour at room temperature in 100 ml of PBS containing 4%

(w/v) BSA to saturate remaining unbound sites. Then antibodies were added and incubation continued overnight. The antibody-containing solution was removed and the filter washed 6 times with 100 ml volumes of PBS. The filter was then incubated for 2 hours in 4% (w/v) BSA dissolved in PBS containing 10^4 cpm [^{125}I]-protein A. All unbound protein A was removed by washing with PBS containing 1% (v/v) Triton-X 100. The filter was then dried and autoradiographed at room temperature using an intensifying screen as described in Section 2.7.5.

Positions of radioactive bands on "Western" blots were determined from the corresponding autoradiographs. The areas with bound iodinated protein were cut from the nitrocellulose filter and placed in scintillation vials. Beckman NA scintillation fluid (4 ml) was added and the vials counted in a LKB Minibeta 1212 scintillation counter.

2.5.4 Analysis of proteins by rocket immunoelectrophoresis

Rocket immunoelectrophoresis (R.I.E.) was performed using a Shandon 600 electrophoresis chamber with methods modified from Laurell (1966) and Plumley & Schmidt (1983). A 1% (w/v) solution of agarose (Sigma, type 1 low EEO) in barbital buffer (Sigma; prepared according to the manufacturers' instructions) containing 1% (v/v) Triton-X 100 was prepared by heating to 100°C and subsequently cooled to, and maintained at, 50°C. The desired antiserum was added and the gels cast on clean, dry microscope slides. A volume of 2

ml of agarose was poured onto each slide. Routinely 125 µl of anti-LSU-binding protein antiserum and 200 µl of anti-RUBISCO antiserum were added to 10 ml of 1% (w/v) agarose. After the agarose had solidified, sample holes (4 mm diameter) were punched along one short edge of the slides. The sample volume loaded into the holes was 10 µl. Slides were placed in the tank and 500 ml of barbital buffer containing 1% (v/v) Triton-X 100 poured into each of the electrode chambers. Strips of Whatman 3MM chromatography paper soaked in barbital buffer were used to form a contact between the gel and the buffer. Electrophoresis was performed at 90 volts for 16 hours at room temperature. Subsequently, slides were removed, the gels washed with PBS and pressed flat under 6 layers of Whatman 3MM chromatography paper to remove uncomplexed proteins. Dried gels were stained for 30 minutes in the following solution:

0.25% (w/v) Coomassie brilliant blue R
50% (v/v) methanol
7% (v/v) acetic acid

Excess stain was removed by immersing the gels in a solution containing 40% (v/v) methanol and 7% (v/v) acetic acid. The area under the rockets was determined by projecting at constant magnification the rocket image onto graph paper, marking the rocket outline and counting the number of squares under the peak. A standard curve of protein amount against rocket area was plotted for each experiment performed.

2.5.5 Ouchterlony immunodiffusion

Sera were tested by the double immunodiffusion technique described by Oudin (1980). A 1% (w/v) solution of agarose in PBS was prepared by heating to 100°C. The agarose was allowed to cool to 50°C and then gels were cast on clean, dry microscope slides. Four wells (diameter 4 mm) were punched around a central well. The serum under test (10 µl) was placed in the central well while test solutions were placed in the surrounding wells. The slides were incubated overnight at room temperature in a closed box containing moist filter paper. After incubation the gels were washed in PBS and pressed flat under 6 layers of Whatman 3MM chromatography paper to remove uncomplexed protein. The gels were dried and stained by immersion in the following solution:

0.25% (w/v) Coomassie brilliant blue R
50% (v/v) methanol
7% (v/v) acetic acid

Excess stain was removed by washing gels in 40% (v/v) methanol; 7% (v/v) acetic acid. The gels were then photographed.

2.6 ANALYSIS OF PROTEINS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

2.6.1 Electrophoresis under denaturing conditions

The method of Laemmli (1970) was followed using slab gels of dimensions 15 cm x 15 cm x 0.15 cm.

Gels (15%) were prepared by mixing together the following:

30% (w/v) acrylamide; 0.8% (w/v) bisacrylamide	24 ml
3 M Tris-HCl (pH 8.8)	6 ml
H_2O	17.4 ml
10% (w/v) SDS	0.48 ml
TEMED	20 µl

Just before casting the gel 200 µl of a freshly prepared 10% (w/v) ammonium persulphate (APS) solution was added. The gel was overlaid with butan-1-ol until polymerisation was complete.

Linear gradient gels (10%-30%) were prepared using a M.S.E. gradient maker (100 ml chamber size). The solutions were prepared as follows:

	10%	30%
low bisacrylamide[a]	-	11 ml
high bisacrylamide[b]	3.65 ml	-
75% (v/v) glycerol	-	8.03 ml
3 M Tris-HCl (pH 8.8)	2.75 ml	2.75 ml
10% (w/v) SDS	0.22 ml	0.22 ml
TEMED	7 µl	9 µl
10% (w/v) APS	53 µl	16 µl

(a) low bisacrylamide contained 60 g acrylamide + 0.3 g bisacrylamide per 100 ml.

(b) high bisacrylamide contained 60 g acrylamide + 1.6 g bisacrylamide per 100 ml.

The freshly prepared APS was added immediately prior to placing each solution in the gradient maker chambers. The gel was overlaid with butan-1-ol until polymerisation was complete. The butan-1-ol was washed off with water and a stacking gel containing the following was cast on top of resolving gels:

30% (w/v) acrylamide; 0.8% (w/v) bisacrylamide	4 ml
0.5 M Tris-HCl (pH 6.8)	5 ml
H_2O	10.8 ml
10% (w/v) SDS	0.2 ml
TEMED	15 µl

Freshly prepared 10% (w/v) APS (100 µl) was added just before

casting the gel and a slot former was inserted before polymerisation. After polymerisation the slot former was removed and the gel mounted in a gel tank. Sample solutions were added to an equal volume of sample buffer and boiled for 4 minutes. The composition of sample buffer was:

0.5 M Tris-HCl (pH 6.8)	2.5 ml
glycerol	2 ml
10% (w/v) SDS	4 ml
2-mercaptoethanol	1 ml
bromophenol blue	0.1 g
H_2O	0.5 ml

Suitable volumes of samples were loaded and the gel was run at 12 mA constant current for 17 hours in a buffer containing:

25 mM Tris base
192 mM glycine
0.1% (w/v) SDS
pH = 8.3

2.6.2 Electrophoresis under non-denaturing conditions

The method of Hedrick and Smith (1968) was employed and 5% slab gels (15 cm x 15 cm x 0.15 cm) were run. The gel solution was prepared as follows:

20% (w/v) acrylamide; 0.8% (w/v) bisacrylamide	15 ml
3 M Tris-HCl (pH 8.8)	7.5 ml
H_2O	35 ml

This was degassed and TEMED (40 µl) and 10% (w/v) APS (420 µl) were added prior to casting the gel. A slot former was inserted before the gel polymerised. No stacking gel was present.

Linear gradient gels (4%-30%) were prepared using a M.S.E. gradient maker (100 ml chamber size). The solutions were prepared as follows:

	4%	30%
low bisacrylamide[a]	-	14.400 ml
high bisacrylamide[b]	4.60 ml	-
30% (v/v) glycerol	-	10.625 ml
3 M Tris-HCl (pH 8.8)	3.75 ml	3.75 ml
H_2O	20.40 ml	-
TEMED	160 µl	75 µl
10% (w/v) APS	17.5 µl	7.5 µl

(a) low bisacrylamide contained 60 g acrylamide + 0.3 g bisacrylamide per 100 ml.

(b) high bisacrylamide contained 25 g acrylamide + 1.25 g bisacrylamide per 100 ml.

The freshly prepared APS was added immediately prior to

placing each solution in the gradient maker chambers. A slot former was inserted before the gel polymerised. No stacking gel was present. After polymerisation the slot former was removed and the gel mounted in the tank. All non-denaturing gels were prerun at 14 mA constant current for 2 hours with the following buffer:

50 mM Tris base
0.384 mM glycine
pH = 8.5
(8 mM L-cysteine was present in
the upper reservoir buffer)

Sufficient glycerol solution (75% (v/v)) containing 1% (w/v) bromophenol blue was added to sample solutions to give a 10% (v/v) solution of glycerol. Samples were loaded and gradient gels were run for a further 20 hours at 18 mA constant current while 5% gels were run for a further 17 hours at 14 mA constant current.

2.6.3 Staining of polyacrylamide gels

Proteins were visualised by immersion of polyacrylamide gels in the following solution for 1 hour:

0.25% (w/v) Coomassie brilliant blue R
50% (v/v) methanol
7% (v/v) acetic acid

Excess stain was removed by washing the gel in a number of changes of 40% (v/v) methanol; 7% (v/v) acetic acid. The gels were then photographed and dried.

For a greater sensitivity in detection of proteins gels were stained by the method of Wray *et al.* (1981). The gel was soaked for 4 hours in at least 4 changes of 50% (v/v) methanol. The staining solution was prepared immediately before use by mixing together solutions A and B as follows:

solution A: 0.8 g silver nitrate dissolved in 4 ml H_2O

solution B: 21 ml of 0.36% (w/v) NaOH and 1.4 ml of 14.8 M ammonium hydroxide.

After mixing the volume was made up to 100 ml with distilled water. The gel was stained with shaking for 15 minutes. The gel was then washed twice with distilled water for 5 minutes and the stain was developed in the following solution which was prepared immediately before use:

2.5 ml 1% (w/v) citric acid
0.25 ml 38% (v/v) formaldehyde
497 ml H_2O

Stain development took 30 minutes. Once development was complete the proteins were fixed by soaking the gel in a solution of 40% (v/v) methanol; 7% (v/v) acetic acid. The gels were then photographed and dried.

2.6.4 Electroelution of proteins from polyacrylamide gels

Proteins to be electroeluted from SDS gels or non-denaturing gels were visualised by the method employing Coomassie brilliant blue R as described in Section 2.6.3. The gel pieces containing the protein were cut from the gel using a scalpel and the pieces placed in glass tubing (diameter 7 mm; length 12 cm). The gel pieces were supported by a plug of polyacrylamide gel. A piece of dialysis tubing knotted at one end and filled with the appropriate buffer was then fixed to the bottom of the glass tubing and the tubing filled with SDS gel running buffer (Section 2.6.1) or non-denaturing gel running buffer (Section 2.6.2) as appropriate. The glass tubing complete with dialysis tubing was then set up in an electrophoresis tank containing the appropriate buffer and electroelution carried out for 17 hours at a constant voltage of 90 volts. The dialysis tubing was then removed from the bottom of the glass tube and the eluted protein in solution was removed by pipette.

2.6.5 Determination of radioactivity in proteins subjected to non-denaturing electrophoresis

The method used was that described by Barraclough & Ellis (1979). Bands corresponding to RUBISCO and the LSU-binding protein complex were removed from stained, dried gels with a scalpel. The bands were placed in destain solution (40% (v/v) methanol; 7% (v/v) acetic acid) until it was possible to remove the Whatman 3MM backing paper. Each

gel piece was then placed in a plastic scintillation vial with 0.2 ml H_2O_2 (100 vol), the vial stoppered and maintained at 70°C for 24 hours. The vials were cooled and Beckman EP scintillation fluid (4 ml) added before counting in a LKB Minibeta 1212 scintillation counter.

2.7 GENERAL TECHNIQUES

2.7.1 Protein assay

Protein was assayed by the dye-binding method of Bradford (1976). Protein solution containing 20 µg to 100 µg of protein in a volume of 100 µl was pipetted into chromic acid-washed test tubes. To this was added 5 ml of protein assay dye reagent concentrate (Biorad), which was diluted according to the manufacturers' instructions. The mixture was vortexed and allowed to stand for 30 minutes. The absorbance at 595 nm was measured using a Shimadzu spectrophotometer and absolute protein concentrations in the unknowns determined from a standard curve prepared with gamma globulins (Sigma).

2.7.2 Chlorophyll assay

Chlorophyll was extracted from membranes prepared as described in Section 2.2. To the membrane pellet was added 3 ml of 90% (v/v) acetone and the extraction was allowed to proceed for 30 minutes in the dark. The debris was removed by centrifugation at top speed in a MSE Minor bench top

centrifuge for 5 minutes and the supernatant removed for assay. The assay used was that of Arnon (1949). The absorbance of the supernatant was determined at 663 nm and 645 nm in quartz cuvettes against a blank containing 90% (v/v) acetone. The concentration of chlorophyll in the extract was determined from the following equation:

$$(A_{645} \times 20.2) + (A_{663} \times 8.02) = [\text{chlorophyll}] \text{ in } \mu g\ ml^{-1}$$

2.7.3 RUBISCO assay

The RUBISCO activity in pea extracts and purified RUBISCO preparations was determined using the method of Lorimer et al. (1977) as follows. Between 50 µl and 100 µl of the enzyme extract was incubated in an Eppendorf tube at 30°C for 5 minutes with 30 µl of $NaHCO_3$ (167 mM), 150 µl of assay buffer (containing 100 mM Tris-HCl, 16 mM $MgCl_2.6H_2O$, pH 8.2) and 5 µCi of $NaH^{14}CO_3$ (56 mCi $mmol^{-1}$). The reaction was started by the addition of 50 µl of ribulose-1,5-bisphosphate (4 mM). Incubation continued at 30°C for up to 10 minutes and was terminated by the addition of 100 µl of 12 M formic acid. An aliquot (200 µl) of the incubation mixture was evaporated to dryness in a glass scintillation vial and the radiolabelled phosphoglyceric acid dissolved in water (1 ml). Beckman EP scintillation fluid (10 ml) was added and the vial counted in a LKB Minibeta 1212 scintillation counter. Efficiency of counting was 90%.

2.7.4 Sucrose density gradient centrifugation

Stromal extracts prepared from light-grown peas and total soluble extracts from dark-grown plants were analysed on sucrose step gradients (5% to 50% (w/v) sucrose). The gradients were composed of 4 x 3 ml steps (5%, 20%, 35% and 50% (w/v) sucrose) and the sucrose solutions were carefully layered one on top of the other using a flow inducer (Watson Marlow Ltd.) The sucrose was dissolved in the following buffer:

50 mM Tris-HCl
7 mM 2-mercaptoethanol
1 mM PMSF
pH = 7.6

If required, ATP (10 mM) and $MgCl_2$ (10 mM) were included in the buffer. The sample (500 ul) was layered on top of the 5% (w/v) sucrose solution and the gradients centrifuged at 88,000 x g for 17 hours at 4°C in a Beckman SW40Ti rotor. Gradients were fractionated using a pipette and fractions (500 µl) were stored at -20°C until required.

2.7.5 Autoradiography

Dried gels containing L-[^{35}S]-methionine-labelled polypeptides and nitrocellulose filters with bound

iodinated proteins from "Western" blotting were exposed to Fuji X-ray film at room temperature. When nitrocellulose filters were autoradiographed Ilford intensifying screens were used.

Film was developed in Kodak LX-24 and fixed in Kodak FX-40 which were both prepared according to the manufacturers' instructions. The film was then washed in tap water and dried.

2.7.6 Photography

Autoradiographs and stained polyacrylamide gels were were placed on an illuminated light box and photographed on Panatomic X film (Kodak). Film was developed in Acutol (Paterson Photographic Ltd.) for 10 minutes and fixed for 10 minutes in Kodak Kodafix (both solutions were prepared according to the manufacturers' instructions). The negatives were washed with water and dried. Prints were prepared on Kodak Kodabrome paper which was developed in Ilford Contrast FF and fixed in Kodafix.

2.8 CHEMICALS

All chemicals were of the highest grade commercially available. Suppliers of specific reagents were as follows:

Amersham International plc, Amersham, Buckinghamshire: L-[^{35}S]-methionine (1000 Ci mmol^{-1}), NaH^{14}CO$_3$

(56 mCi $mmol^{-1}$), $Na^{125}I$ (13.5 mCi μg^{-1}).

B.D.H. Chemicals Ltd., Poole, Dorset:
Acrylamide, ammonium persulphate, sodium dodecyl sulphate.

Eastman Kodak, Rochester, New York, USA:
N,N'-methylene bisacrylamide, N,N,N,N'-tetramethylene diamine.

Pharmacia (GB) Ltd., London:
Sephacryl S300 superfine, DEAE Sephacel, cyanogen bromide-activated Sepharose, low molecular weight protein kit for denaturing polyacrylamide gels, PD-10 desalting columns.

Sigma Chemical Co. Ltd., Poole, Dorset:
Adenosine triphosphate (ATP), ribulose-1,5-bisphosphate (RuBP), phenylmethylsulphonyl fluoride (PMSF), Coomassie brilliant blue R, agarose (type 1 low EEO), barbital buffer, gamma globulins (bovine), bovine serum albumin (BSA), Staphylococcus aureus Protein A, chloramine T, L-methionine, L-cysteine.

SECTION THREE - RESULTS AND DISCUSSION

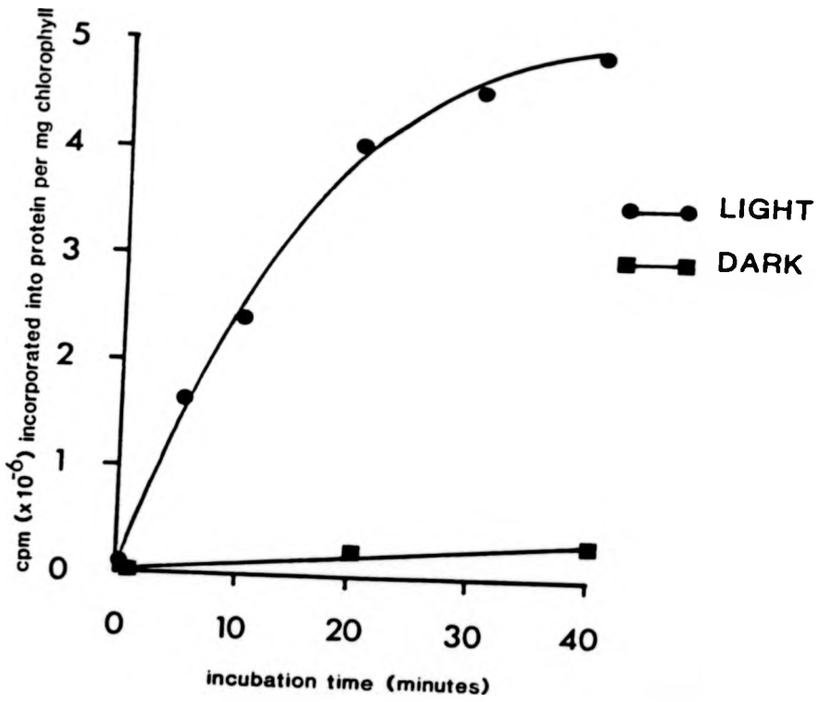
3.1 CHARACTERISATION OF THE RUBISCO LSU-BINDING PROTEIN

3.1.1 Radiolabelling of chloroplast proteins

Chloroplasts possess their own DNA (Ris & Plaut, 1962) and all the enzymes necessary for protein synthesis (reviewed by Boulter *et al.*, 1972). Isolated intact chloroplasts will incorporate radiolabelled amino-acids into proteins if incubated in the light (Blair & Ellis, 1973; Bottomley *et al.*, 1974; Fish & Jagendorf, 1982). Intact isolated pea chloroplasts have been shown to carry out initiation, elongation and termination of polypeptide chains (Highfield & Ellis, 1976) and have been shown to be permeable to most amino-acids (Nobel & Wang, 1970). In such subcellular systems the energy for protein synthesis is supplied by photosynthetic phosphorylation. Therefore the chloroplasts used need not be rigorously purified from other subcellular organelles since the latter cannot use light to form the ATP necessary to drive protein synthesis. The same is true of broken chloroplasts present in the preparation. It is important, however, to ensure that there is no bacterial contamination of the chloroplast preparation to be incubated with radiolabelled amino-acids as this can lead to incorporation of radioactivity into bacterial protein. A determination of the extent of radiolabelled amino-acid incorporation in the dark is a simple test for bacterial contamination.

Figure 3.1 shows a time course of radiolabelled amino-

Figure 3.1 - Incorporation of [^{35}S]-methionine into chloroplast proteins. Chloroplasts (95 µg of chlorophyll) were isolated as described in Section 2.3.1 and incubated with 25 µCi of [^{35}S]-methionine for 40 minutes at 20°C and light intensity of 100 µE m^{-2} s^{-1}. Aliquots (5 µl) were removed and pipetted onto filter paper for determination of acid-insoluble counts (see Section 2.3.2). The amount of incorporation of [^{35}S]-methionine is expressed per milligram of chlorophyll.



acid incorporation into chloroplast proteins. The incorporation rate is initially high but falls markedly after 30 minutes. This decrease in incorporation has been observed by other workers (Blair & Ellis, 1973; Bottomley et al., 1974; Fish & Jagendorf, 1982). This cessation of incorporation is not due to chloroplast lysis (Ellis, 1977).

3.1.2 The association of binding protein with newly-synthesised large subunits of RUBISCO

It has been shown that the major soluble product of chloroplast protein synthesis is the large subunit (LSU) of RUBISCO (Blair & Ellis, 1973; Hartley et al., 1975; Bottomley et al., 1974). In Pisum sativum the gene encoding the LSU polypeptide has been characterised and its position mapped on chloroplast DNA (Oishi & Tewari, 1983). Studies with spinach have shown that newly-synthesised LSU will comigrate with holoenzyme on 5% non-denaturing polyacrylamide gels, suggesting that there is sufficient small subunit (SSU) available in isolated spinach chloroplasts to allow RUBISCO assembly to proceed (Bottomley et al., 1974).

To investigate the fate of newly-synthesised LSU in Pisum sativum chloroplasts, soluble products of chloroplast protein synthesis were subjected to electrophoresis under non-denaturing conditions. An autoradiograph showing the labelled proteins present is presented in Figure 3.2. Two major labelled bands are visible. Under identical conditions

Figure 3.2 - Labelling of RUBISCO and RUBISCO large subunit-binding protein complex *in vitro*. Isolated chloroplasts (950 µg of chlorophyll) were incubated with 250 µCi of [^{35}S]-methionine for up to 90 minutes at 20°C under a light intensity of 100 µE m^{-2} s^{-1}, as described in Section 2.3.2. At intervals aliquots (200 µl) were removed and centrifuged to pellet the chloroplasts. Chloroplasts were lysed in a buffer containing 25 mM Tris base, 0.0192 mM glycine and 6.7 mM methionine and centrifuged to remove membranes. Aliquots (100 µl) of supernatant solution were electrophoresed on a 5% non-denaturing gel as described in Section 2.6.2. The gel was stained, dried and exposed to X-ray film for 72 hours. The figure shows the positions of RUBISCO and the RUBISCO LSU-binding protein complex on the autoradiograph.

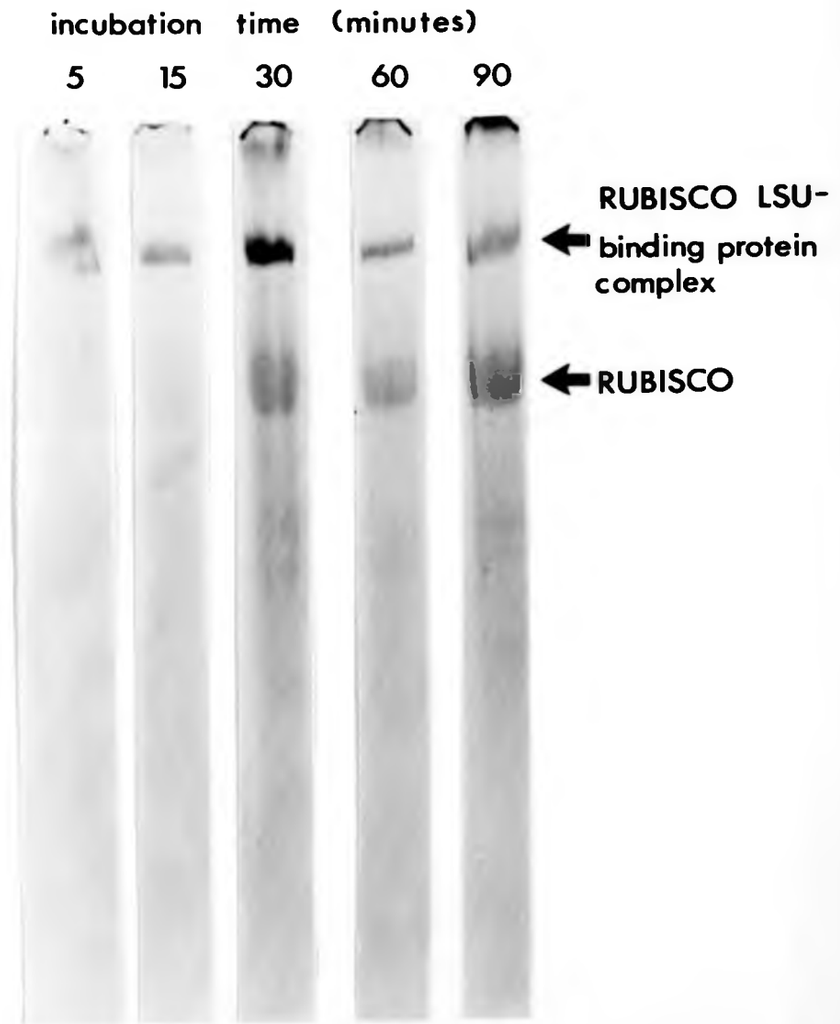
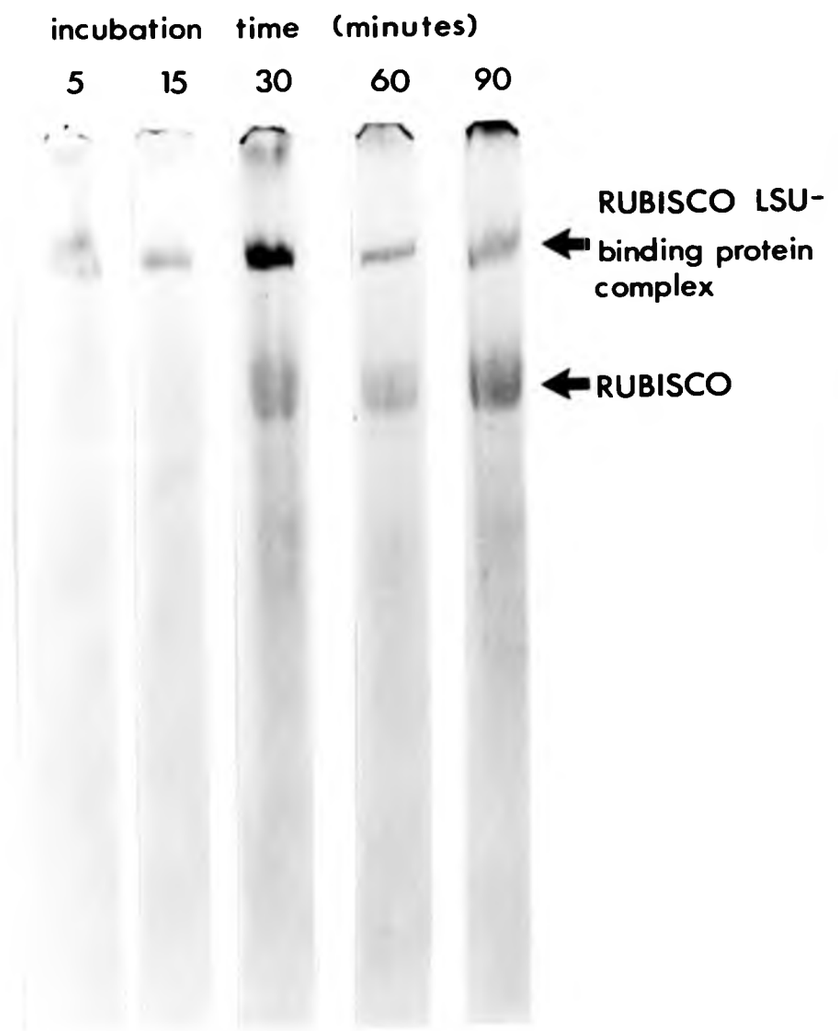


Figure 3.2 - Labelling of RUBISCO and RUBISCO large subunit-binding protein complex *in vitro*. Isolated chloroplasts (950 µg of chlorophyll) were incubated with 250 µCi of [^{35}S]-methionine for up to 90 minutes at 20°C under a light intensity of 100 µE m^{-2} s^{-1}, as described in Section 2.3.2. At intervals aliquots (200 µl) were removed and centrifuged to pellet the chloroplasts. Chloroplasts were lysed in a buffer containing 25 mM Tris base, 0.0192 mM glycine and 6.7 mM methionine and centrifuged to remove membranes. Aliquots (100 µl) of supernatant solution were electrophoresed on a 5% non-denaturing gel as described in Section 2.6.2. The gel was stained, dried and exposed to X-ray film for 72 hours. The figure shows the positions of RUBISCO and the RUBISCO LSU-binding protein complex on the autoradiograph.



other workers have identified the faster-migrating of the two bands to be RUBISCO (Barraclough & Ellis, 1980). The radioactivity present in the RUBISCO on the non-denaturing gel is due to the presence of newly-synthesised LSU. SSU is not labelled under these conditions since it is synthesised on cytoplasmic ribosomes as a precursor and imported post-translationally (Chua & Schmidt, 1978; Highfield & Ellis, 1978; Smith & Ellis, 1979). It is clear from Figure 3.2 that some assembly of LSU into RUBISCO holoenzyme is occurring. For such an assembly to proceed there must be pools of unassembled SSU present in the stroma of the isolated chloroplasts. The existence of such a pool has been demonstrated in leaves of Pisum (Roy et al., 1978b) and in this pool the SSU is present as a monomer (Roy et al., 1979).

The presence of a second major labelled protein is evident from Figure 3.2. This slower-migrating band was examined further by electroelution from the non-denaturing gel and subsequent electrophoresis into a second dimension SDS gel. The band is composed of two types of subunit as shown in Figure 3.3. Only one of these is labelled; this comigrates with the LSU of RUBISCO and represents newly-synthesised but unassembled LSU. The second subunit present migrates more slowly than LSU and is unlabelled, suggesting it is cytoplasmic in origin. This protein was initially characterised by Barraclough & Ellis (1980) and termed the RUBISCO LSU-binding protein by Roy et al. (1982). The LSU-binding protein has been shown to be

Figure 3.3 - The association of newly-synthesised LSUs of RUBISCO with RUBISCO large subunit-binding protein. Isolated chloroplasts were incubated with [^{35}S]-methionine for 15 minutes at 20°C and with a light intensity of 100 µE m^{-2} s^{-1}. Chloroplasts were lysed in 10 mM Tris-HCl, pH 8.0 and centrifuged to remove membranes as described in Section 2.3.2. Aliquots (50 µl) of the supernatant solution were subjected to electrophoresis under non-denaturing conditions. The gel was stained to locate the RUBISCO large subunit-binding protein complex which was excised from the gel and electroeluted into a buffer containing 25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3 as described in Section 2.6.4. The electroeluted protein was subjected to electrophoresis under denaturing conditions and this second dimension gel was stained, dried and exposed to X-ray film for 1 week.

(A) represents the first dimension non-denaturing gel; (B) and (C) represent denaturing gels.
Lane markings are as follows: (a) stained stromal protein; (b) autoradiograph of (a); (c) stained molecular weight markers; (d) stained stromal proteins; (e) electroeluted RUBISCO LSU-binding protein complex (stained); (f) autoradiograph of (d); (g) autoradiograph of (e).
(complex) represents the position of the RUBISCO LSU-binding protein complex on the non-denaturing gel; (BP) represents the position of the RUBISCO LSU-binding protein subunit on the denaturing gel.

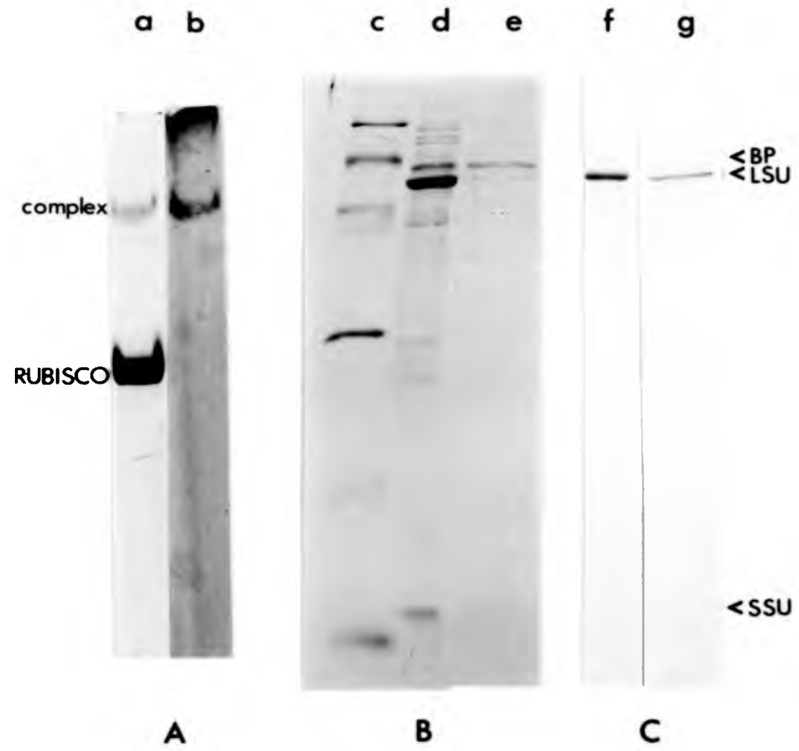
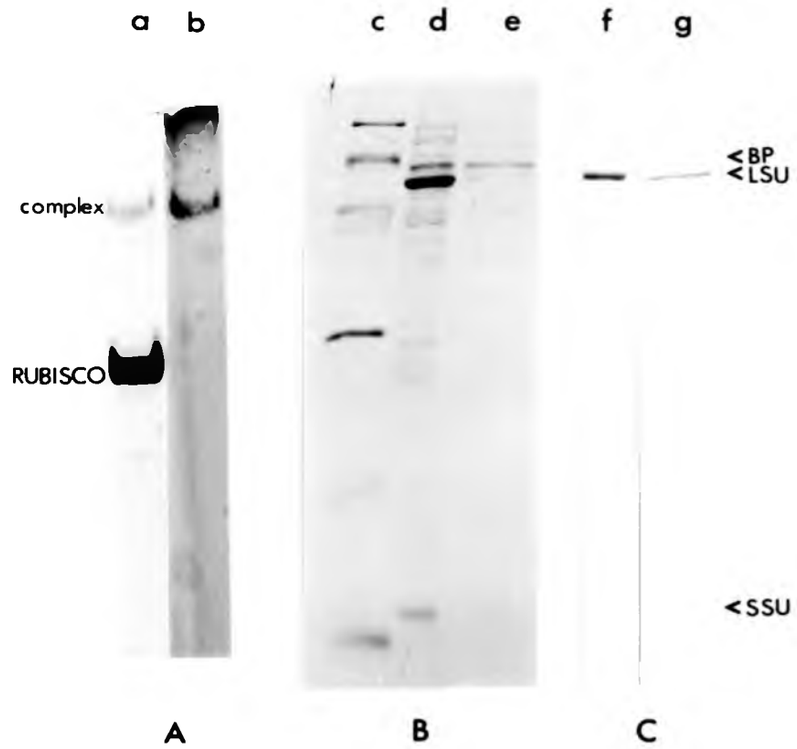


Figure 3.3 - The association of newly-synthesised LSUs of RUBISCO with RUBISCO large subunit-binding protein. Isolated chloroplasts were incubated with [^{35}S]-methionine for 15 minutes at 20°C and with a light intensity of 100 µE m^{-2} s^{-1}. Chloroplasts were lysed in 10 mM Tris-HCl, pH 8.0 and centrifuged to remove membranes as described in Section 2.3.2. Aliquots (50 µl) of the supernatant solution were subjected to electrophoresis under non-denaturing conditions. The gel was stained to locate the RUBISCO large subunit-binding protein complex which was excised from the gel and electroeluted into a buffer containing 25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3 as described in Section 2.6.4. The electroeluted protein was subjected to electrophoresis under denaturing conditions and this second dimension gel was stained, dried and exposed to X-ray film for 1 week.

(A) represents the first dimension non-denaturing gel; (B) and (C) represent denaturing gels.
Lane markings are as follows: (a) stained stromal protein; (b) autoradiograph of (a); (c) stained molecular weight markers; (d) stained stromal proteins; (e) electroeluted RUBISCO LSU-binding protein complex (stained); (f) autoradiograph of (d); (g) autoradiograph of (e).
(complex) represents the position of the RUBISCO LSU-binding protein complex on the non-denaturing gel; (BP) represents the position of the RUBISCO LSU-binding protein subunit on the denaturing gel.



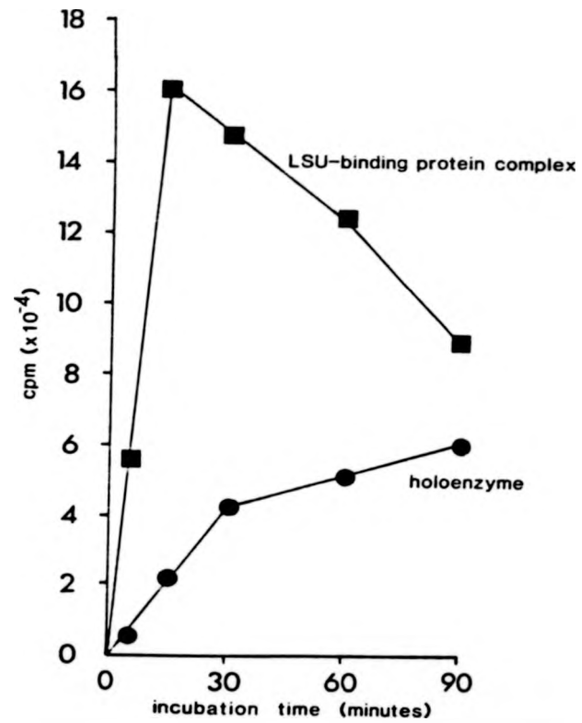
synthesised on poly A$^+$ mRNA as a high molecular weight precursor (Hemmingsen & Ellis, 1986). The relative numbers of each of the two subunits, LSU and the RUBISCO LSU-binding protein, present in the slowly migrating complex shown in Figure 3.2 is unclear and this question of stoichiometry will be addressed below in Section 3.2.3.

This co-migration of newly-synthesised LSU with the binding protein does not appear to be accidental (Hemmingsen & Ellis, 1986). It was noted that the stained complex on non-denaturing gels and the radiolabelled band on an autoradiograph from the same gel always superimpose exactly (see Figure 3.3, tracks a and b), suggesting that the complex has radioactivity uniformly associated with it. This suggests that the RUBISCO LSU-binding protein complex is not composed of binding protein subunits and newly-synthesised LSU which fortuitously comigrate on non-denaturing gels. The binding protein appears to be associated specifically with the newly-synthesised LSU in the chloroplast stroma; analysis of stromal protein on sucrose density gradients has shown that few, if any, proteins co-sediment with the LSU-binding protein complex (Roy et al., 1982).

The presence of newly-synthesised LSU in this complex led Barraclough & Ellis (1980) to propose the hypothesis that the RUBISCO LSU-binding protein complex may be an intermediate in the assembly of RUBISCO. As shown in Figure 3.2, the intensity of labelling in the RUBISCO holoenzyme increases with time of incubation whereas the label in the LSU-binding protein complex decreases in intensity. The bands

corresponding to both RUBISCO holoenzyme and the RUBISCO LSU-binding protein complex were excised from the non-denaturing gel of Figure 3.2 and their radioactivity measured. Figure 3.4 shows that as the time of incubation proceeds the amount of label in the holoenzyme increases whilst that of the slower-migrating LSU-binding protein complex falls. The increase in radioactivity in RUBISCO holoenzyme after 30 minutes is not due to continued amino-acid incorporation since chloroplasts cease to incorporate labelled amino-acids into protein after 30 minutes (Figure 3.1). In the prolonged incubation illustrated in Figure 3.2, therefore, the events following LSU synthesis can be studied. Pulse-chase studies carried out by Roy et al., (1982) have shown that, in isolated Pisum chloroplasts, newly-synthesised LSU can be assembled into holoenzyme during the chase period. Only the LSU-binding protein complex was labelled during the pulse period, which was of 30 minutes duration. The results presented in Figures 3.2 and 3.4 are in accord with those of Barraclough & Ellis (1980) who found that, for the initial 30 minutes of chloroplast incubation, most of the radiolabelled LSU migrated not with the RUBISCO holoenzyme on a non-denaturing gel but with the LSU-binding protein complex. From these data, it is evident that the assembly of newly-synthesised LSU into holoenzyme in isolated chloroplasts is slow, occurring 30 minutes after incorporation of radiolabelled amino-acids has begun. This may reflect the non-physiological nature of the experimental system; there is no continual input of SSU, for

Figure 3.4 - Assembly of RUBISCO large subunit into holoenzyme. Bands corresponding to RUBISCO and RUBISCO large subunit-binding protein complex were excised from the gel used to produce the autoradiograph shown in Figure 3.2. Bands were counted as described in Section 2.6.5, to obtain an estimate of [^{35}S]-methionine incorporation into each protein. Counts were corrected for background. holoenzyme = RUBISCO, LSU-binding protein complex = RUBISCO LSU-binding protein complex.



instance. In vivo labelling experiments with Pisum plants by Roy et al. (1982) have shown that a labelling time of 15 minutes is sufficient to ensure that radiolabelled SSU is incorporated into holoenzyme although incorporation of LSU takes longer. Radiolabelled LSU appeared in holoenzyme only when the in vivo labelling time was extended beyond 15 minutes. Therefore a delay in incorporation of LSU into holoenzyme may be a feature of the RUBISCO assembly reaction.

The LSU-binding protein complex may have a role to play in donating newly-synthesised LSU for RUBISCO holoenzyme assembly but Barraclough & Ellis (1980) have noted that in non-denaturing gel analyses of labelled chloroplast proteins there is radioactive LSU present at the top of the gel, and so a precursor-product relationship cannot be inferred with certainty. This immobile radioactivity is also seen in Figure 3.2 and its presence may be responsible for the discrepancy between the drop in counts associated with the LSU-binding protein complex and the rise in counts associated with the RUBISCO holoenzyme (Figure 3.4). There has been a report recently of a chloroplast ATP-dependent protease which will digest proteins which have been newly-synthesised in isolated chloroplasts (Liu & Jagendorf, 1984). This protease may be responsible for a loss of radioactivity associated with the LSU. The role of the LSU-binding protein complex in holoenzyme assembly has been investigated by Milos & Roy (1984) and these data will be discussed more fully in Section 4. The data presented in this

section confirm the observations of Barraclough & Ellis (1980). The next step was to purify the RUBISCO LSU-binding protein complex so that the binding protein could be characterised and antibodies prepared.

3.2 PURIFICATION AND PROPERTIES OF THE RUBISCO LSU-BINDING PROTEIN COMPLEX

3.2.1 Purification of RUBISCO and the RUBISCO LSU-binding protein complex

RUBISCO, by virtue of its abundance and high molecular weight, is a protein which can be easily and quickly purified. The carboxylase has been crystallised from protein extracts prepared from Triticum, Spinacia and Nicotiana and is readily purified by sucrose density gradient centrifugation (reviewed by Ellis, 1979).

The purification procedure adopted here, however, was chosen to allow an initial co-purification of RUBISCO and the RUBISCO LSU-binding protein complex. It has been estimated (Ellis, 1977) that the Mr of the complex is in excess of 600,000, whereas RUBISCO has a Mr of approximately 560,000 (Kung, 1976). To purify two such high molecular weight proteins, gel filtration chromatography was employed in conjunction with ion exchange chromatography. The method followed was modified from that of Hemmingsen & Ellis (1986). The gel filtration medium used was Sephacryl S300 superfine, chosen because the two proteins of interest were within its

quoted fractionation range (10,000 - 1,500,000). The rigid gel matrix allows high flow rates to be employed and the small bead size ensures good resolution.

Figure 3.5 shows the absorbance at 280 nm of the fractions eluting from the S300 column. The peak represents the position of RUBISCO; this is verified by the coincident peak of CO_2-fixing activity. The electrophoretic mobilities of eluted proteins is shown in Figure 3.6. It is clear that RUBISCO is the major protein. The RUBISCO LSU-binding protein complex eluted three or four fractions before the fraction containing the peak of the eluted RUBISCO and this separation allowed the binding protein-containing fractions to be pooled, with removal of much of the RUBISCO.

The contaminating RUBISCO was removed from the RUBISCO LSU-binding protein complex, and RUBISCO was further purified by ion exchange chromatography. The resin chosen was DEAE-Sephacel, which is available in the pre-swollen state and is therefore easy to pack into a column. The latter can be operated at a high flow rate. A batch elution of protein from the column followed loading. This method of elution was chosen in preference to elution by a salt gradient since high amounts of RUBISCO were loaded. Hence it was possible to utilise a column of large bed volume and so purify large quantities. Batch-wise elution also facilitated speedy recovery of any RUBISCO LSU-binding protein complex which was present in the RUBISCO-containing fractions from the S300 column, as this remained bound to the DEAE-Sephacel after RUBISCO elution.

Figure 3.5 - Elution profile from the Sephacryl 300 column illustrated by absorbance at 280 nm. Stromal protein was precipitated with ammonium sulphate and loaded onto the S300 column as described in Section 2.4. Fractions were collected immediately and the absorbance at 280 nm of each fraction determined. Selected fractions were assayed for RUBISCO activity by measurement of $^{14}CO_2$ fixed into acid-insoluble material as described in Section 2.7.3.

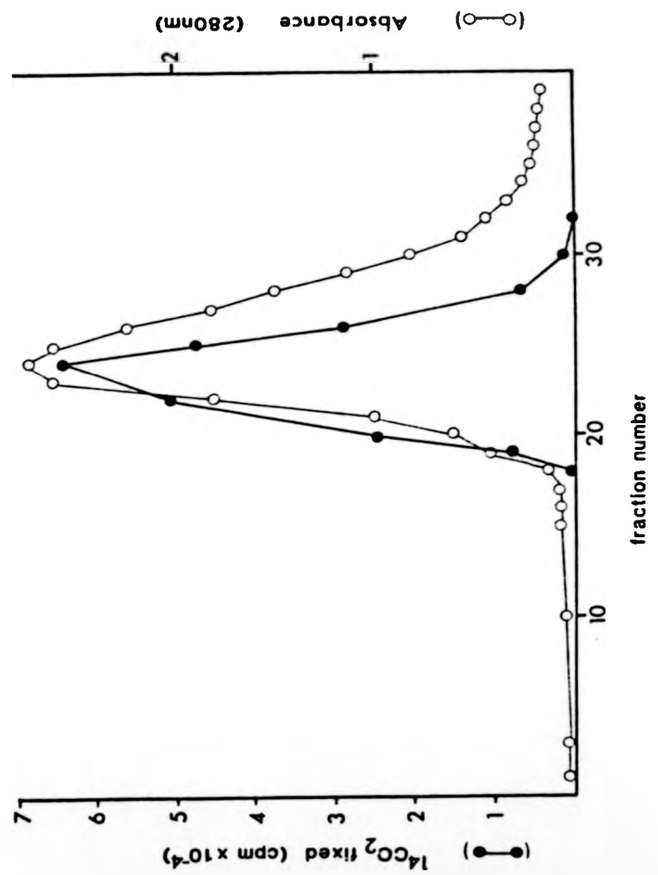


Figure 3.6 - Elution profile from the Sephacryl 300 column illustrated by SDS polyacrylamide gel electrophoresis. Stromal protein was precipitated with ammonium sulphate and loaded onto the S300 column as described in Section 2.4. Aliquots of selected fractions were loaded onto a 15% SDS polyacrylamide gel. After electrophoresis the proteins were visualised by Coomassie blue staining. BP = RUBISCO LSU-binding protein subunit, LSU = RUBISCO large subunit, SSU = RUBISCO small subunit, STR = stromal extract.

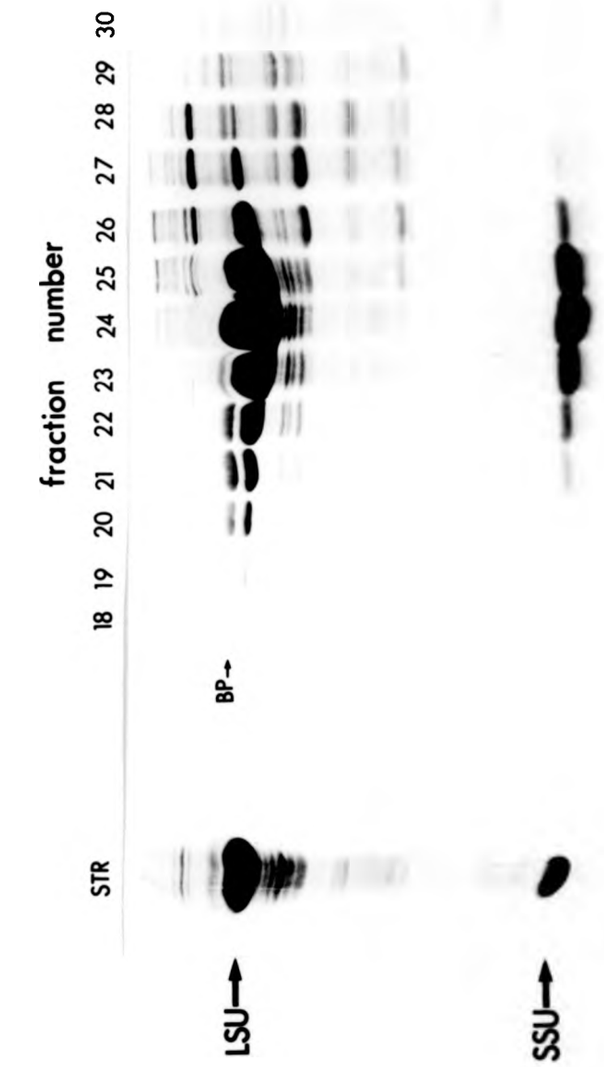
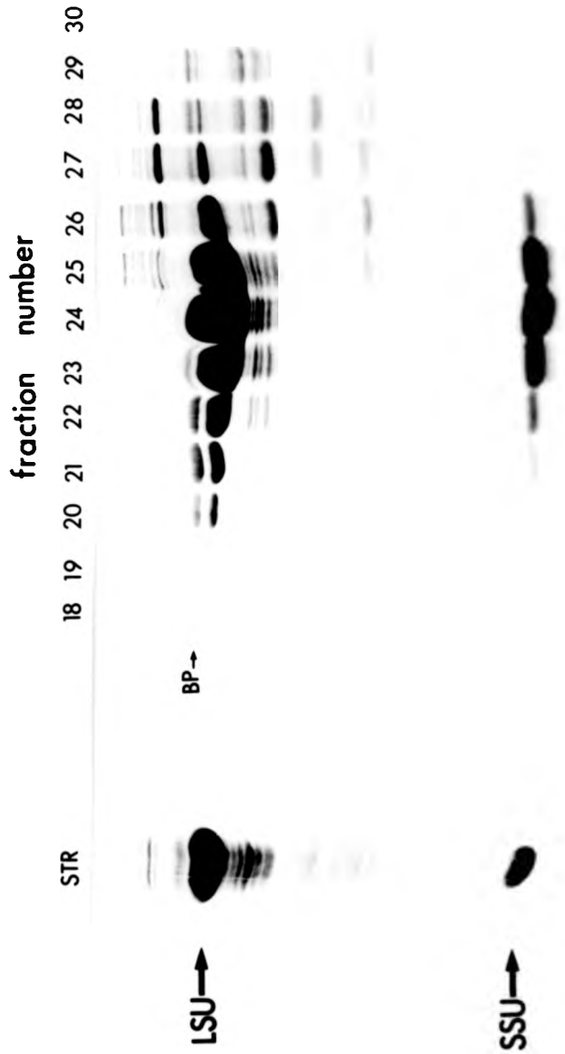


Figure 3.6 - Elution profile from the Sephacryl 300 column illustrated by SDS polyacrylamide gel electrophoresis. Stromal protein was precipitated with ammonium sulphate and loaded onto the S300 column as described in Section 2.4. Aliquots of selected fractions were loaded onto a 15% SDS polyacrylamide gel. After electrophoresis the proteins were visualised by Coomassie blue staining. BP = RUBISCO LSU-binding protein subunit, LSU = RUBISCO large subunit, SSU = RUBISCO small subunit, STR = stromal extract.



A second round of ion exchange chromatography was usually necessary for complete purification of RUBISCO. The purity of RUBISCO at different stages in the purification was determined by subjecting the protein to electrophoresis under denaturing and non-denaturing conditions. Representative gels are shown in Figures 3.7 and 3.8. Purified RUBISCO, as visualised by silver-staining a 15% SDS polyacrylamide gel is shown in Figure 3.9. After elution from the DEAE-Sephacel column, the RUBISCO LSU-binding protein complex was also subjected to further ion exchange chromatography on a column of 8 ml bed volume both to ensure complete purification and to reduce the volume of the eluted protein. The purity of the LSU-binding protein complex was also determined at different stages in the purification by subjecting the protein to electrophoresis under denaturing and non-denaturing conditions. Representative gels are shown in Figures 3.7 and 3.8. Purified LSU-binding protein complex, as visualised by silver-staining is shown in Figure 3.9. The silver-stained gel shows that the RUBISCO LSU-binding protein complex has a small amount of associated LSU. The presence of this LSU is due to RUBISCO contamination. There is no SSU visible because the silver staining method differentially detects the RUBISCO subunits at low loadings of RUBISCO (see Section 3.3.2).

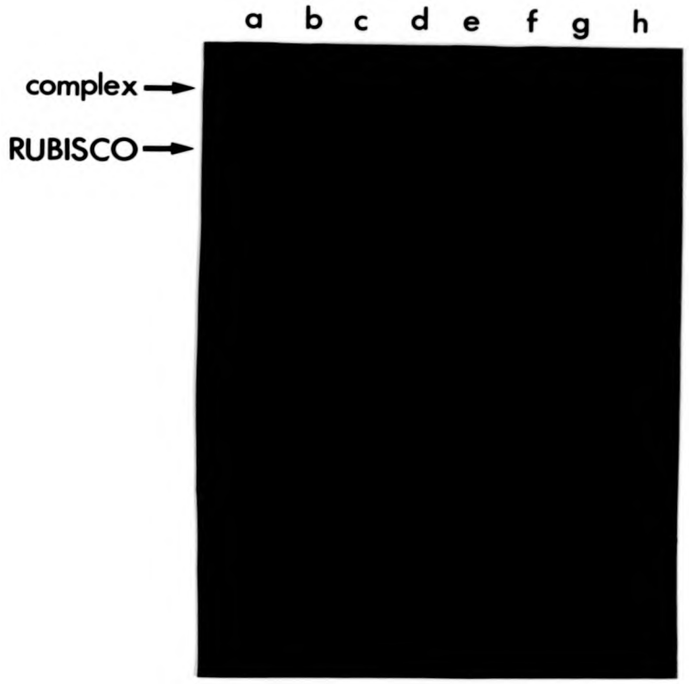
The yields obtained for RUBISCO and the RUBISCO LSU-binding protein complex from 300 g of 10-day old Pisum plants were typically 100 mg and 5 mg respectively. Plant extracts contain between 1 mg and 10 mg of RUBISCO per g of fresh weight (Ellis, 1979). With Nicotiana the RUBISCO

Figure 3.7 - Purification of RUBISCO and RUBISCO large subunit-binding protein complex illustrated by non-denaturing electrophoresis. Samples of protein at various stages throughout the purification were analysed by electrophoresis under non-denaturing conditions. Proteins were visualised by staining with Coomassie blue as described in Section 2.6.3.

Lane markings are as follows: (a) and (e) stromal extract; (b) RUBISCO large subunit-binding protein complex peak from the S300 column; (c) RUBISCO large subunit-binding protein complex from the first round of ion-exchange chromatography; (d) RUBISCO large subunit-binding protein complex from the second round of ion-exchange chromatography; (f) RUBISCO peak from the S300 column; (g) RUBISCO from the first round of ion-exchange chromatography; (h) RUBISCO from the second round of ion-exchange chromatography. complex = RUBISCO LSU-binding protein complex.

Figure 3.7 - Purification of RUBISCO and RUBISCO large subunit-binding protein complex illustrated by non-denaturing electrophoresis. Samples of protein at various stages throughout the purification were analysed by electrophoresis under non-denaturing conditions. Proteins were visualised by staining with Coomassie blue as described in Section 2.6.3.

Lane markings are as follows: (a) and (e) stromal extract; (b) RUBISCO large subunit-binding protein complex peak from the S300 column; (c) RUBISCO large subunit-binding protein complex from the first round of ion-exchange chromatography; (d) RUBISCO large subunit-binding protein complex from the second round of ion-exchange chromatography; (f) RUBISCO peak from the S300 column; (g) RUBISCO from the first round of ion-exchange chromatography; (h) RUBISCO from the second round of ion-exchange chromatography. complex = RUBISCO LSU-binding protein complex.



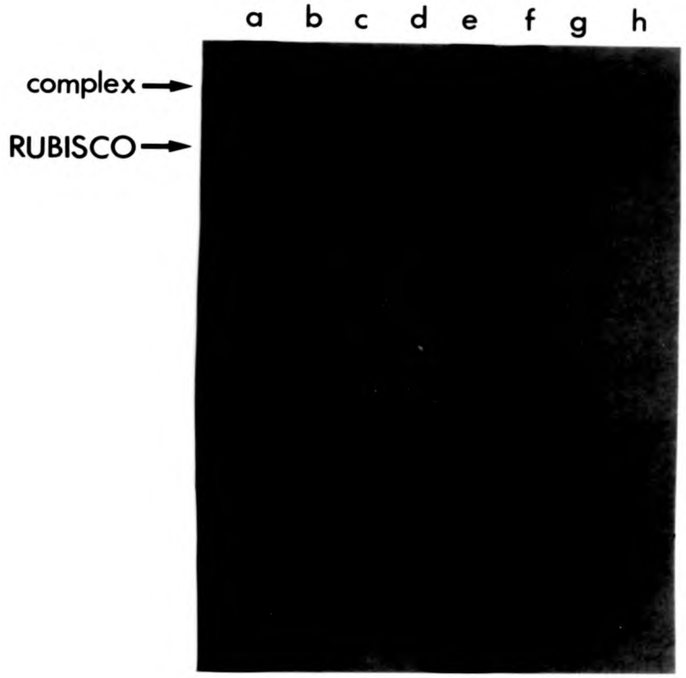


Figure 3.7 - Purification of RUBISCO and RUBISCO large subunit-binding protein complex illustrated by non-denaturing electrophoresis. Samples of protein at various stages throughout the purification were analysed by electrophoresis under non-denaturing conditions. Proteins were visualised by staining with Coomassie blue as described in Section 2.6.3.

Lane markings are as follows: (a) and (e) stromal extract; (b) RUBISCO large subunit-binding protein complex peak from the S300 column; (c) RUBISCO large subunit-binding protein complex from the first round of ion-exchange chromatography; (d) RUBISCO large subunit-binding protein complex from the second round of ion-exchange chromatography; (f) RUBISCO peak from the S300 column; (g) RUBISCO from the first round of ion-exchange chromatography; (h) RUBISCO from the second round of ion-exchange chromatography. complex = RUBISCO LSU-binding protein complex.

Figure 3.8 - Purification of RUBISCO and RUBISCO large subunit-binding protein complex illustrated by electrophoresis under denaturing conditions. Samples of protein at various stages throughout the purification were analysed by electrophoresis under denaturing conditions. Proteins were visualised by staining with Coomassie blue.

Lane markings are as follows: (a) and (j) molecular weight markers; (b) and (f) stromal extract; (c) RUBISCO peak from S300 column; (d) RUBISCO from first round of ion-exchange chromatography; (e) RUBISCO from second round of ion-exchange chromatography; (g)RUBISCO large subunit-binding protein peak from S300 column; (h) RUBISCO large subunit-binding protein complex from first round of ion-exchange chromatography; (i) RUBISCO large subunit-binding protein complex from second round of ion-exchange chromatography.
(1) position of binding protein subunit; (2) position of LSU; (3) position of SSU.

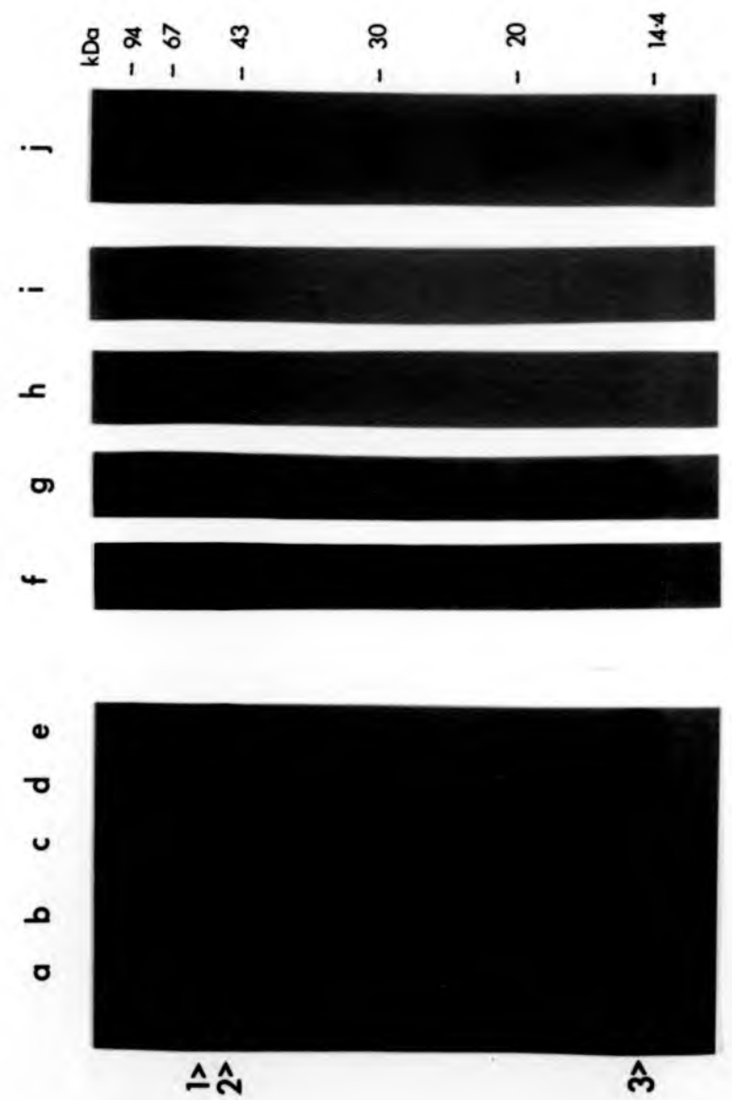


Figure 3.8 - Purification of RUBISCO and RUBISCO large subunit-binding protein complex illustrated by electrophoresis under denaturing conditions. Samples of protein at various stages throughout the purification were analysed by electrophoresis under denaturing conditions. Proteins were visualised by staining with Coomassie blue.

Lane markings are as follows: (a) and (j) molecular weight markers; (b) and (f) stromal extract; (c) RUBISCO peak from S300 column; (d) RUBISCO from first round of ion-exchange chromatography; (e) RUBISCO from second round of ion-exchange chromatography; (g)RUBISCO large subunit-binding protein peak from S300 column; (h) RUBISCO large subunit-binding protein complex from first round of ion-exchange chromatography; (i) RUBISCO large subunit-binding protein complex from second round of ion-exchange chromatography.

(1) position of binding protein subunit; (2) position of LSU; (3) position of SSU.

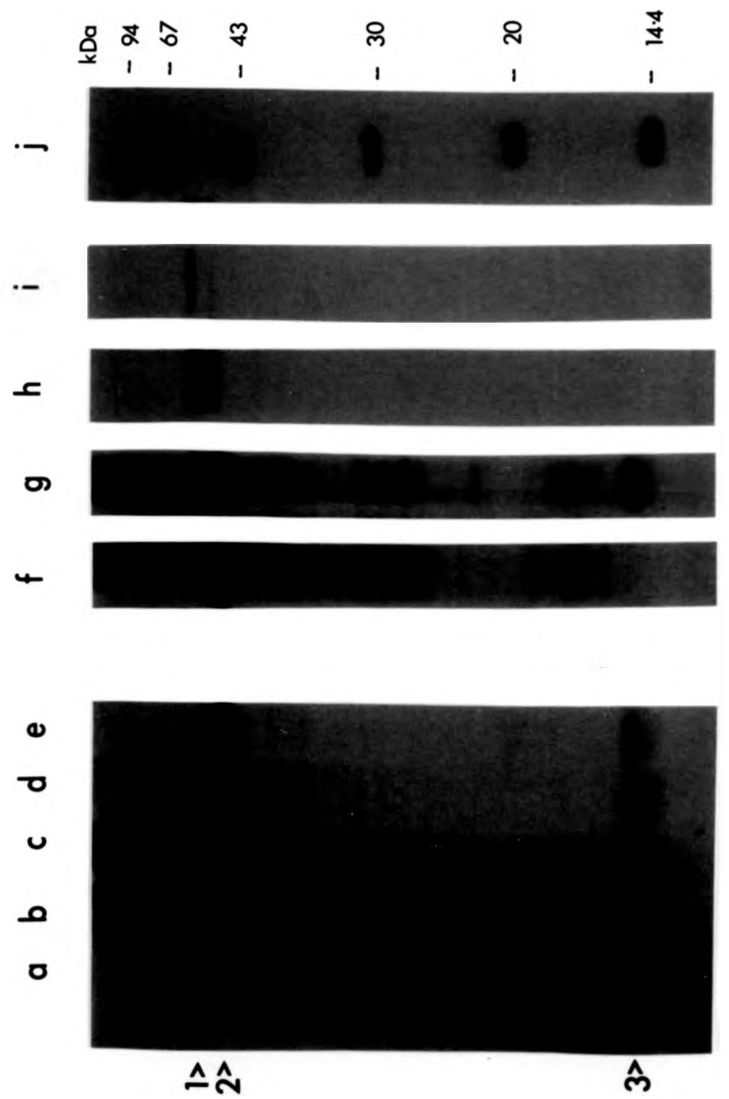


Figure 3.9 - Purity of preparations of RUBISCO and RUBISCO large subunit-binding protein complex. The purity of both proteins was determined by silver staining as described in Section 2.6.3. Purified proteins were subjected to electrophoresis under denaturing conditions on a 15% polyacrylamide gel.

Proteins were loaded as follows: lane (A) purified RUBISCO; lane (B) purified RUBISCO large subunit-binding protein complex; lane (C) molecular weight markers.

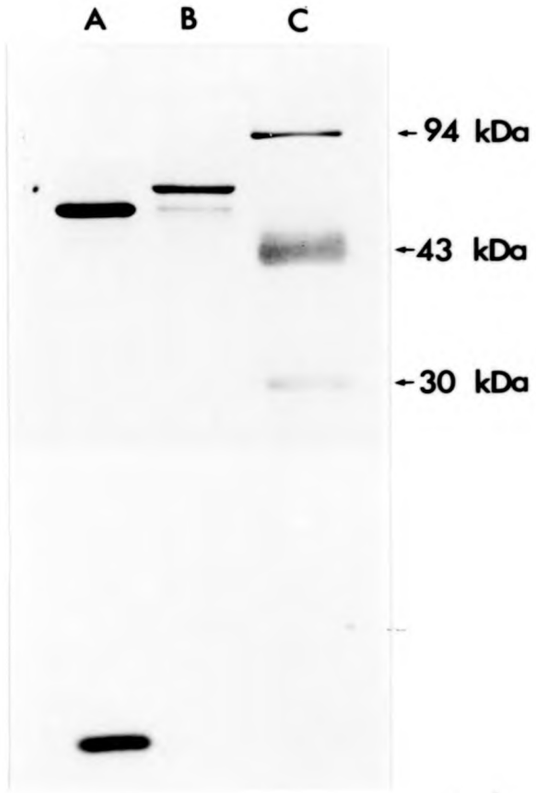
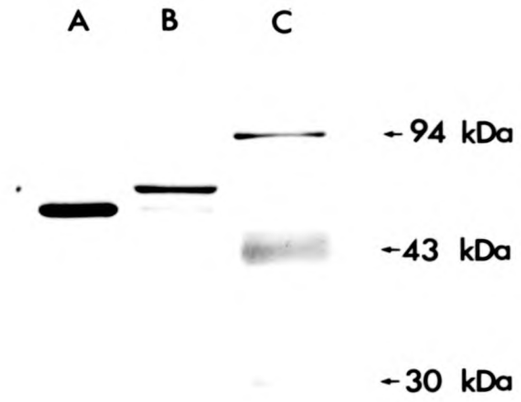


Figure 3.9 - Purity of preparations of RUBISCO and RUBISCO large subunit-binding protein complex. The purity of both proteins was determined by silver staining as described in Section 2.6.3. Purified proteins were subjected to electrophoresis under denaturing conditions on a 15% polyacrylamide gel.

Proteins were loaded as follows: lane (A) purified RUBISCO; lane (B) purified RUBISCO large subunit-binding protein complex; lane (C) molecular weight markers.



yield is about 4 mg per gram of fresh leaf tissue (Kung, 1976). The yield obtained using the purification method described above is 0.4 mg per gram of 10-day old leaf tissue. At this age the leaves are still expanding and synthesising further RUBISCO, but as the tissue ages, increased starch reserves make isolation of intact chloroplasts difficult. A good chloroplast preparation is therefore favoured at the expense of yield. The LSU-binding protein yield is, as expected from Figure 3.6, approximately twenty-fold lower than that obtained for RUBISCO. It is possible that hydrophobic interactions are involved in maintenance of the quaternary structure of the complex (Hemmingsen & Ellis, 1986) and that at 4°C, the temperature at which the purification was performed, some complex molecules have dissociated into subunits which would elute after RUBISCO. This problem could be overcome by purification at higher temperature. The protocol served well in the rapid preparation of sufficient pure RUBISCO and RUBISCO LSU-binding protein complex for the immunisation of rabbits.

3.2.2 Purification and characterisation of antibodies

Antibodies were used to study the expression of the RUBISCO LSU-binding protein genes and of the genes for the subunits of RUBISCO, at the protein level, on illumination of etiolated plants. In particular, the absolute contents of both RUBISCO and the LSU-binding protein were determined by rocket immunoelectrophoresis, a technique requiring specific

antisera. The material used to immunise rabbits is shown on the polyacrylamide gel presented in Figure 3.9. RUBISCO and RUBISCO LSU-binding protein complex were used in the native form for immunisation, for reasons that will be discussed below. The method used for raising these antisera is described in Section 2.5.1.

The specificity of the resulting antibody raised against RUBISCO LSU-binding protein complex was evaluated initially by the Ouchterlony double-diffusion technique (Oudin, 1980). This method is based on the migration of individual molecules through an agarose gel, their migration rates depending upon their initial concentrations. When antibody interacts with antigen a large macromolecular aggregate is formed which cannot diffuse further. This precipitate will prevent further diffusion of the particular antigen involved but not of another non-identical antigen. Figure 3.10 shows the reaction of crude serum raised against the RUBISCO LSU-binding protein complex with both a stromal extract and the purified complex. Two separate precipitin bands form in each reaction. If identical antigens are placed in wells around a central well containing antibody, an arc will form between adjacent wells. This identity is seen in Figure 3.10; the purified complex is a stromal component. The cross reaction with two proteins, both in the stromal extract and in the RUBISCO LSU-binding protein complex was confirmed by a "Western" blot analysis of gels on which samples of RUBISCO, stromal extract and RUBISCO LSU-binding protein complex had been run. The resulting autoradiograph is shown

in Figure 3.11. The anti-binding protein antiserum reacts with both LSU-binding protein and the large subunit of RUBISCO. This was to be expected since the native complex used to elicit antibodies has a small amount of contaminating RUBISCO large subunit associated with it.

It was necessary to remove this cross reaction and affinity chromatography was the method of choice. It was decided to link purified RUBISCO to a Sepharose column and using this, to remove the contaminating antibody, leaving the filtered serum clean and ready for use. This method, rather than one employing a RUBISCO LSU-binding protein-Sepharose conjugate, was preferred for two reasons. Firstly, sufficient RUBISCO LSU-binding protein free of LSU would be difficult to obtain and, secondly, release of bound LSU-binding protein antiserum would require the use of urea or other such chaotropic reagents, a treatment which can interfere with subsequent immunological assays (Plumley & Schmidt, 1983). After subjection to affinity chromatography, the filtered serum was free of any cross-reactivity with RUBISCO large subunit, as judged by immunodiffusion (Figure 3.10) and "Western" blots (Figure 3.11). The anti-binding protein serum reactedonly with the binding protein subunits.

The serum raised against RUBISCO, when tested against a stromal extract and purified RUBISCO, reacted strongly with large subunit but poorly with small subunit (Figure 3.12). There was, however, no cross-reaction with binding protein subunits in the stromal extract, or indeed any other stromal component. This lack of antigenicity of the small subunit has

Figure 3.10 - Characterisation of antibodies raised against the RUBISCO large subunit-binding protein complex as determined by Ouchterlony immunodiffusion. Stromal proteins and purified RUBISCO large subunit-binding protein complex were placed in wells surrounding a central well containing either crude serum or serum which had been purified by affinity chromatography (see Section 2.5.1). Diffusion was allowed to proceed for 16 hours at 4°C, as described in Section 2.5.5, and then plates were washed in PBS, dried and immunoprecipitates visualised by staining with Coomassie blue.

Well groups 1, 2, 3 & 4 were loaded identically.
(A) represents the cross-reaction obtained using crude serum;
(B) represents the cross-reaction obtained with affinity-purified serum.
In each group
Wells contained the following: (a) and (c) stromal extracts;
(b) purified RUBISCO large subunit-binding protein complex.

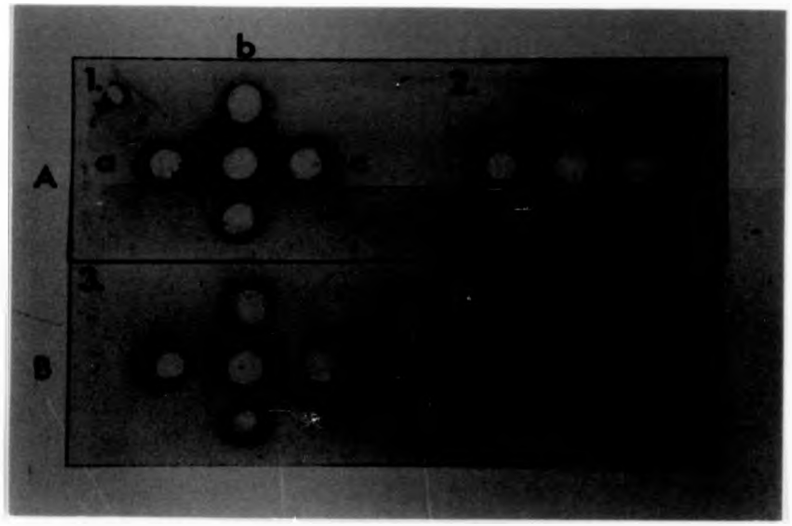


Figure 3.10 - Characterisation of antibodies raised against the RUBISCO large subunit-binding protein complex as determined by Ouchterlony immunodiffusion. Stromal proteins and purified RUBISCO large subunit-binding protein complex were placed in wells surrounding a central well containing either crude serum or serum which had been purified by affinity chromatography (see Section 2.5.1). Diffusion was allowed to proceed for 16 hours at 4°C, as described in Section 2.5.5, and then plates were washed in PBS, dried and immunoprecipitates visualised by staining with Coomassie blue.

Well groups 1, 2, 3 & 4 were loaded identically.
(A) represents the cross-reaction obtained using crude serum;
(B) represents the cross-reaction obtained with affinity-purified serum.
In each group
Wells contained the following: (a) and (c) stromal extracts;
(b) purified RUBISCO large subunit-binding protein complex.

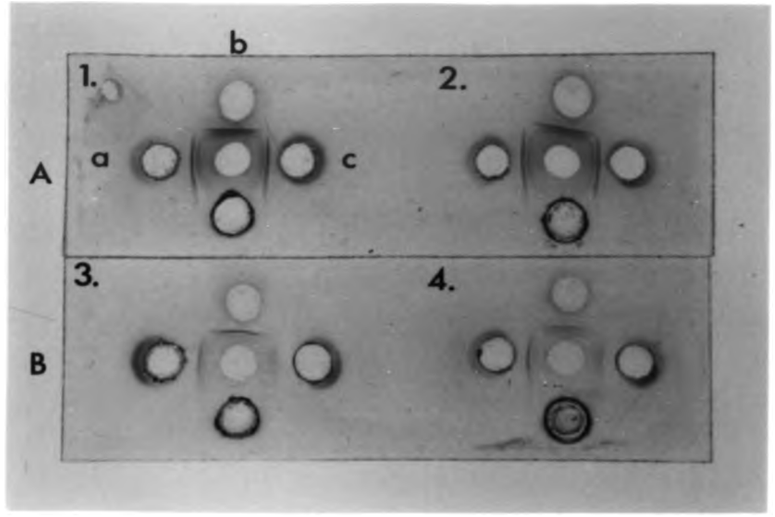


Figure 3.10 - Characterisation of antibodies raised against the RUBISCO large subunit-binding protein complex as determined by Ouchterlony immunodiffusion. Stromal proteins and purified RUBISCO large subunit-binding protein complex were placed in wells surrounding a central well containing either crude serum or serum which had been purified by affinity chromatography (see Section 2.5.1). Diffusion was allowed to proceed for 16 hours at 4°C, as described in Section 2.5.5, and then plates were washed in PBS, dried and immunoprecipitates visualised by staining with Coomassie blue.

Well groups 1, 2, 3 & 4 were loaded identically.
(A) represents the cross-reaction obtained using crude serum;
(B) represents the cross-reaction obtained with affinity-purified serum.
In each group
Wells contained the following: (a) and (c) stromal extracts;
(b) purified RUBISCO large subunit-binding protein complex.

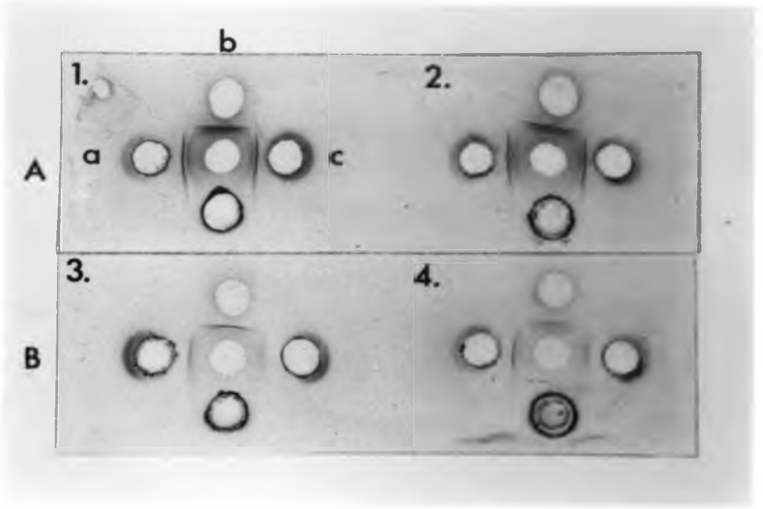


Figure 3.11 - Characterisation of antiserum raised against RUBISCO large subunit-binding protein complex as determined by "Western" blotting. The purified complex was run on a 15% SDS polyacrylamide gel and the proteins were blotted onto nitrocellulose filter as described in Section 2.5.3. The filters were incubated overnight with 4% (w/v) BSA containing either crude antiserum or affinity purified serum. Filters were washed and subsequently incubated with [^{125}I]-protein A (10^4 cpm per filter) before exposure to X-ray film for 16 hours. "BEFORE" represents the cross-reaction obtained using crude serum; "AFTER" represents the cross-reaction obtained using affinity-purified serum. Lane markings are as follows: (a) purified RUBISCO; (b) purified RUBISCO large subunit-binding protein complex; (c) stromal extract. BP represents RUBISCO LSU-binding protein subunit; LSU represents RUBISCO LSU.

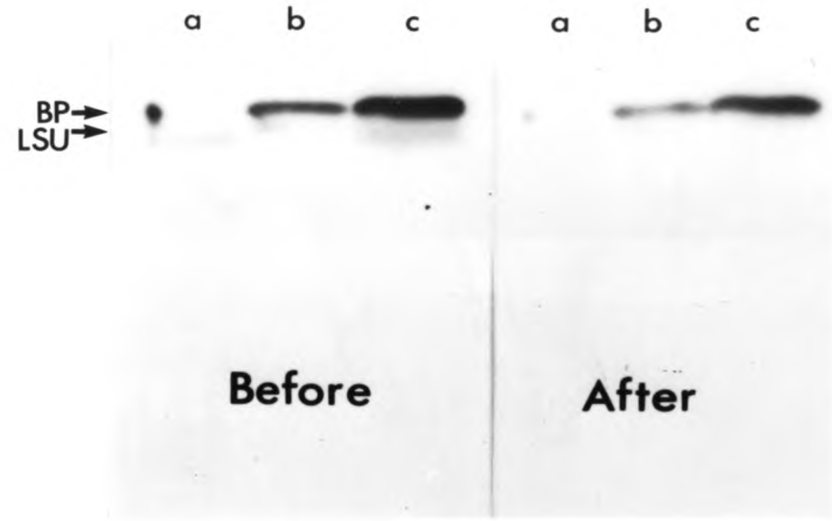


Figure 3.11 - Characterisation of antiserum raised against RUBISCO large subunit-binding protein complex as determined by "Western" blotting. The purified complex was run on a 15% SDS polyacrylamide gel and the proteins were blotted onto nitrocellulose filter as described in Section 2.5.3. The filters were incubated overnight with 4% (w/v) BSA containing either crude antiserum or affinity purified serum. Filters were washed and subsequently incubated with [^{125}I]-protein A (10^6 cpm per filter) before exposure to X-ray film for 16 hours. "BEFORE" represents the cross-reaction obtained using crude serum; "AFTER" represents the cross-reaction obtained using affinity-purified serum. Lane markings are as follows: (a) purified RUBISCO; (b) purified RUBISCO large subunit-binding protein complex; (c) stromal extract. BP represents RUBISCO LSU-binding protein subunit; LSU represents RUBISCO LSU.

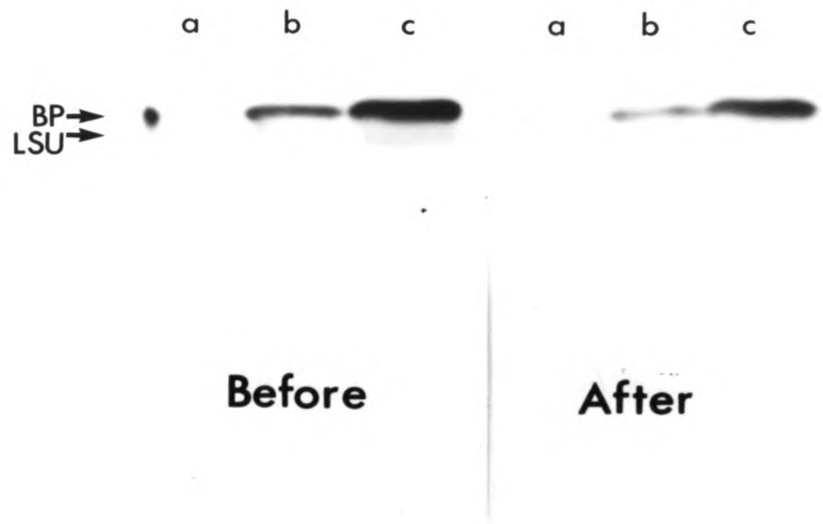


Figure 3.12 - Characterisation of antiserum raised against RUBISCO as determined by "Western" blotting. A 15% SDS gel was loaded with a stromal extract and the gel run overnight. Proteins were blotted onto a nitrocellulose filter, as described in Section 2.5.3, and the filter was incubated overnight with 4% (w/v) BSA containing either antiserum raised against native RUBISCO or the latter with additional antiserum raised against wheat SSU.

Lane markings are as follows: (a) purified RUBISCO; (b) stromal extract.

(1) shows the cross-reaction with crude serum alone; (2) shows the cross-reaction when additional antiserum raised against wheat SSU is present.
(BP) represents the position of the RUBISCO LSU-binding protein subunit as observed on the denaturing gel.

Figure 3.12 - Characterisation of antiserum raised against RUBISCO as determined by "Western" blotting. A 15% SDS gel was loaded with a stromal extract and the gel run overnight. Proteins were blotted onto nitrocellulose filter, as described in Section 2.5.3, and the filter was incubated overnight with 4% (w/v) BSA containing either antiserum raised against native RUBISCO or the latter with additional antiserum raised against wheat SSU.

Lane markings are as follows: (a) purified RUBISCO; (b) stromal extract.

(1) shows the cross-reaction with crude serum alone; (2) shows the cross-reaction when additional antiserum raised against wheat SSU is present.
(BP) represents the position of the RUBISCO LSU-binding protein subunit as observed on the denaturing gel.

been reported elsewhere (Highfield, 1978). It may have been that in the native RUBISCO used for immunisation, the antigenic sites of small subunit were masked. To ensure detection of small subunits on "Western" blots, anti-RUBISCO antiserum was supplemented with anti-small subunit antiserum raised against the SSU prepared from SDS-denatured RUBISCO of Triticum aestivum, kindly provided by Dr. G. Schmidt of Rothamsted Experimental Station. Figure 3.12 (2) shows that reaction of this antisera "cocktail" with purified RUBISCO gave a strong signal with both subunits.

The technique of "Western" blotting was used extensively throughout the course of this work to determine the presence of RUBISCO and binding protein subunits. The technique is simple to use and is both sensitive and specific; if specific immune sera are used, the proteins of interest can be analysed in complex mixtures. The sensitivity of the blotting procedure is a function of the antibody used. It has been reported that levels as low as 1-2 ng of a specific protein can be detected (Burnette, 1981).

The blotting medium used was nitrocellulose, the surface of which is negatively charged at pH 8.0. As proteins are eluted from polyacrylamide gels as anions, adsorbance to the filter involves factors other than electrostatic forces. Nitrocellulose is a convenient medium for blotting as its relatively low capacity for protein binding means that quenching of unbound sites after transfer is both quick and easy (Gershoni & Palade, 1982). The rate and efficiency of transfer depends on the molecular weight of the protein under

study. Lower molecular weight proteins leave the gel faster than higher molecular weight proteins.

As "Western" blots were to be used to estimate changes in amounts of LSU, SSU and binding protein on illumination of etiolated plants, and to examine the distribution of these proteins on sucrose density gradient centrifugation (Section 3.3), it was important to demonstrate that the amounts of radiolabelled protein A bound to the antigen-antibody complexes on the nitrocellulose filter exhibited a linear relationship with the amount of protein which had been subjected to electrophoresis. Linearity has been demonstrated with proteins extracted from Saccharomyces carlsbergensis cells (Vaessen et al., 1981), although in this work linearity was only observed provided that amounts of the protein under study were less than 25 µg. To investigate the nature of the immunodetection of the RUBISCO subunits by "Western" blotting, increasing amounts of purified RUBISCO were subjected to electrophoresis on a 15% SDS polyacrylamide gel and the proteins transferred onto nitrocellulose. The resulting autoradiograph is presented in Figure 3.13. Both LSU and SSU are readily detectable at amounts of RUBISCO of 4.2 µg and above. Below this value LSU is visible but the SSU signal is indistinct. To determine whether a linear relationship existed between counts bound to the nitrocellulose filter and the amount of RUBISCO protein loaded onto the original polyacrylamide gel, a 15% SDS polyacrylamide gel was loaded with purified RUBISCO and blotted. The nitrocellulose filter pieces which corresponded

to the positions of LSU and SSU were excised and counted for radioactivity and the results are shown in Figure 3.14. Linearity was maintained up to levels of 40 µg of RUBISCO and levels as low as 2 µg could be detected. It is evident, however, that at low levels of RUBISCO (<5 µg) and with the antisera "cocktail" used, the RUBISCO LSU was more readily detectable than was the SSU.

It was also important to ensure that no variation across a nitrocellulose filter occurred during the blotting process. To ensure that an even transfer was occurring, identical loadings of a soluble protein extract were applied to a 15% SDS polyacrylamide gel, the gel blotted and bands corresponding to LSU, SSU and binding protein excised and counted. The result is shown in Figure 3.15. Most variability was seen with SSU. In this case the variation was between 19% and 25%. Much lower variability was seen with LSU and binding protein, suggesting that uneven transfer was not an inherent problem of "Western" blotting.

It is important to note that in estimating proteins by counting protein A-labelled antigen-antibody complexes, results from within one immunoblot only were compared. Counts corresponding to the same proteins from different immunoblots cannot be compared because of differences in blotting time, time of incubation with antibody and with radiolabelled protein A. Similarly, counts from different proteins on one immunoblot cannot be compared due to differences in the antibody reactions. However, relative changes in the amount of one protein across one

Figure 3.13 - Quantitation of immunodetection by "Western" blots. Increasing amounts of purified RUBISCO were loaded onto a 15% SDS polyacrylamide gel. After electrophoresis the proteins were blotted onto nitrocellulose filter. This was incubated overnight with 4% (w/v) BSA containing antiserum raised against RUBISCO (100 µl per filter) and wheat anti-SSU antiserum (50 µl per filter). The filter was then incubated with [^{125}I]-protein A (10^6 cpm per filter) and exposed to X-ray film for 16 hours. LSU - position of RUBISCO LSU, SSU - position of RUBISCO SSU.

The amount of RUBISCO loaded onto each track was as follows: (a) 1.4 µg; (b) 2.8 µg; (c) 4.2 µg; (d) 5.6 µg; (e) 7.0 µg; (f) 14.0 µg; (g) 21.0 µg; (h) 28.0 µg; (i) 35.0 µg; (j) 42.0 µg; (k) 70.0 µg; (l) 84.0 µg.

Figure 3.13 - Quantitation of immunodetection by "Western" blots. Increasing amounts of purified RUBISCO were loaded onto a 15% SDS polyacrylamide gel. After electrophoresis the proteins were blotted onto nitrocellulose filter. This was incubated overnight with 4% (w/v) BSA containing antiserum raised against RUBISCO (100 µl per filter) and wheat anti-SSU antiserum (50 µl per filter). The filter was then incubated with [^{125}I]-protein A (10^4 cpm per filter) and exposed to X-ray film for 16 hours. LSU - position of RUBISCO LSU, SSU - position of RUBISCO SSU.

The amount of RUBISCO loaded onto each track was as follows: (a) 1.4 µg; (b) 2.8 µg; (c) 4.2 µg; (d) 5.6 µg; (e) 7.0 µg; (f) 14.0 µg; (g) 21.0 µg; (h) 28.0 µg; (i) 35.0 µg; (j) 42.0 µg; (k) 70.0 µg; (l) 84.0 µg.

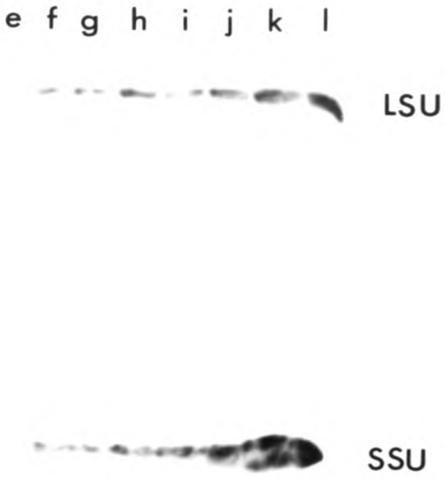


Figure 3.14 - Quantitation of immunodetection of RUBISCO by "Western" blotting. Purified RUBISCO was subjected to electrophoresis on a 15% SDS polyacrylamide gel and the proteins subsequently transferred onto a nitrocellulose filter. The filter was incubated with antiserum raised against RUBISCO LSU and SSU and ^{125}I-labelled protein A to allow detection of both subunits (see Section 2.5.3). The filter was exposed to X-ray film for 16 hours and the autoradiograph used to determine the positions of RUBISCO LSU and SSU on the corresponding nitrocellulose filter. Bands on the filter corresponding to LSU and SSU were excised and counted for radioactivity. All counts were corrected for background, which was obtained from pieces of filter which gave no darkening of film. Counts for LSU and SSU were plotted against the amount of RUBISCO loaded.

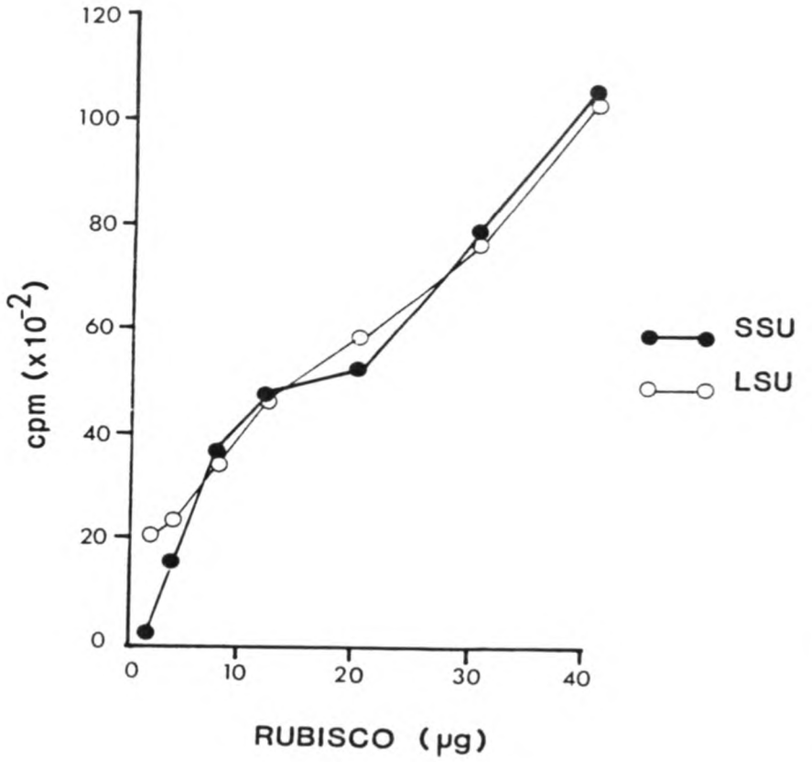
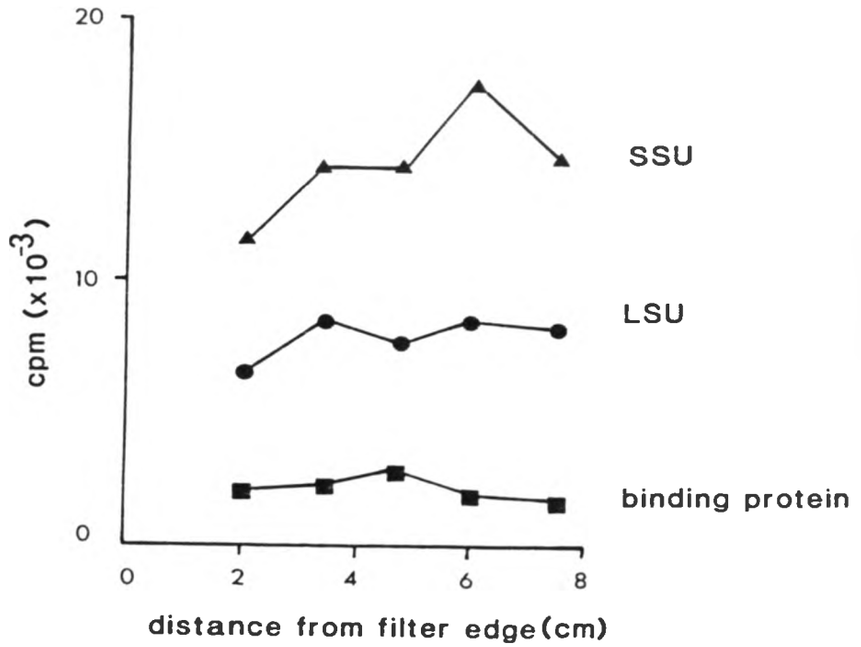


Figure 3.15 - Variability in immunodetection by " Western" blotting. Identical loadings of a stromal extract were run on a 15% SDS polyacrylamide gel and the proteins blotted onto a nitrocellulose filter. This was incubated overnight with 4% (w/v) BSA containing antiserum raised against RUBISCO (100 µl per filter), wheat anti-SSU antiserum (50 µl per filter) and antiserum raised against the RUBISCO large subunit-binding protein complex (100 µl per filter). The filter was then incubated with [^{125}I]-protein A (10^4 cpm per filter) and exposed to X-ray film for 16 hours. Bands on the filter corresponding to LSU and SSU were excised and counted for radioactivity. All counts were corrected for background which was obtained from pieces of filter which gave no darkening of film. Counts for LSU, SSU and binding protein subunit were plotted against the distance of the band from the left hand edge of the filter.



immunoblot can be compared, as a linear relationship was shown to exist between the protein loaded and the counts bound to the filter (Figure 3.14).

A much more accurate method of determining amounts of RUBISCO and binding protein is by rocket immunoelectrophoresis (R.I.E.). This is a powerful analytical technique with great resolving power. As with the "Western" blotting technique, an estimate of the amount of one protein in a complex mixture can be obtained. The method is rapid, unlike single radial immunodiffusion, as it is based on electrophoresis. The agarose gels used contain a uniform concentration of antibody and must be of a uniform thickness. Agarose with low electroendosmotic properties must be employed, as the negative charge of the agarose can generate an electro-osmotic flow of water through the gel which may lead to spurious separation of proteins. On electrophoresis, the antigens behave as anions, and migrate into the gel while the antibodies are cations at the pH used . Initially, soluble antibody-antigen complexes form as there is antigen excess. As the antigen migrates further, it becomes more dilute, as some is held back in antigen-antibody complexes. Once equivalence is reached, an insoluble precipitate is formed which redissolves as more antigen reaches it and the precipitate moves forward. When no further antigen can enter the precipitate, a stable arc is formed which is stationary and usually in the shape of a rocket.

In R.I.E., specific antisera against individual proteins are required. Figure 3.11 shows that the

affinity-purified antiserum raised against RUBISCO LSU-binding protein reacts only with the binding protein; it does not recognise LSU or SSU. Similarly, the anti-RUBISCO is specific for RUBISCO (Figure 3.12). Samples analysed by R.I.E were soluble protein extracts prepared as described in Section 2.2. No SDS was present in the samples as this has been found to solubilise the forming antigen-antibody complexes in R.I.E., although rocket formation in the presence of SDS is possible if Triton, polyethylene glycol and calcium ions are included in the gel (Plumley & Schmidt, 1983). Native proteins were therefore used to elicit antibodies in rabbits rather than SDS-denatured proteins.

Representative rockets obtained with both RUBISCO and binding protein are shown in Figures 3.16 and 3.17. The electrophoresis was performed at 90 volts for 17 hours to ensure equivalence had been reached. The work of Laurell (1966) shows that migration distance is constant over a wide range of antigen concentration if electrophoresis is continued for 6 hours, and that the area under the rocket is proportional to the concentration of antigen. Amounts of RUBISCO and binding protein in the extracts under study were determined from standard curves, representatives of which are shown in Figures 3.18 and 3.19. One curve was prepared for each R.I.E. run performed. For greater accuracy in estimating RUBISCO amounts at low concentrations, a second standard curve was prepared using the results obtained from lower standard RUBISCO concentrations.

The remaining work described in this section concerns

Figure 3.16 - Characterisation by rocket immunoelectrophoresis of antiserum raised against RUBISCO. Increasing amounts of purified RUBISCO were loaded into wells formed in agarose which contained serum raised against native RUBISCO holoenzyme. Electrophoresis was performed at 90 volts for 16 hours as described in Section 2.5.4, after which time the gels were washed with PBS to remove uncomplexed protein, and the immunocomplexes were visualised by Coomassie blue staining.

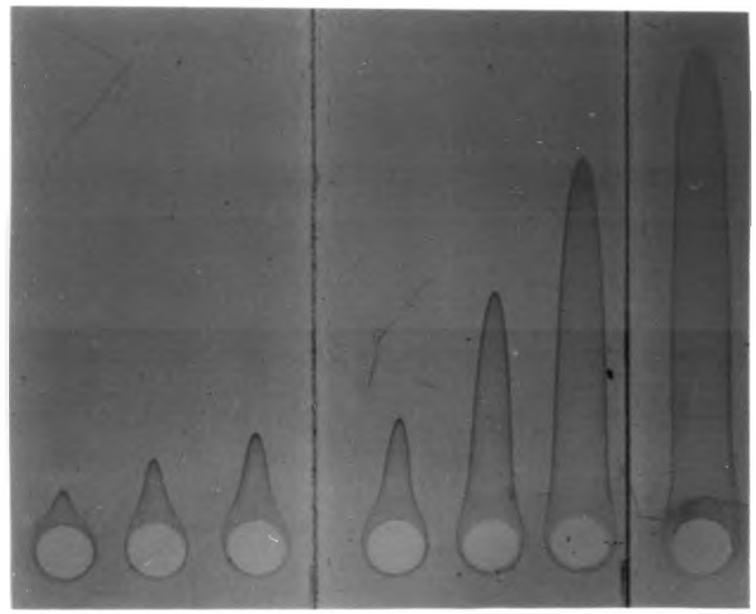


Figure 3.16 - Characterisation by rocket immunoelectrophoresis of antiserum raised against RUBISCO. Increasing amounts of purified RUBISCO were loaded into wells formed in agarose which contained serum raised against native RUBISCO holoenzyme. Electrophoresis was performed at 90 volts for 16 hours as described in Section 2.5.4, after which time the gels were washed with PBS to remove uncomplexed protein, and the immunocomplexes were visualised by Coomassie blue staining.

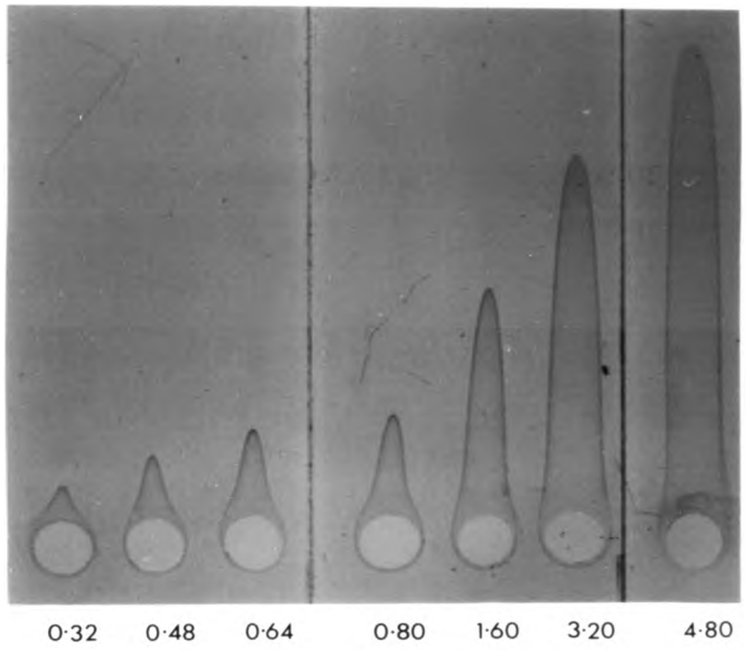


Figure 3.16 – Characterisation by rocket immunoelectrophoresis of antiserum raised against RUBISCO. Increasing amounts of purified RUBISCO were loaded into wells formed in agarose which contained serum raised against native RUBISCO holoenzyme. Electrophoresis was performed at 90 volts for 16 hours as described in Section 2.5.4, after which time the gels were washed with PBS to remove uncomplexed protein, and the immunocomplexes were visualised by Coomassie blue staining.

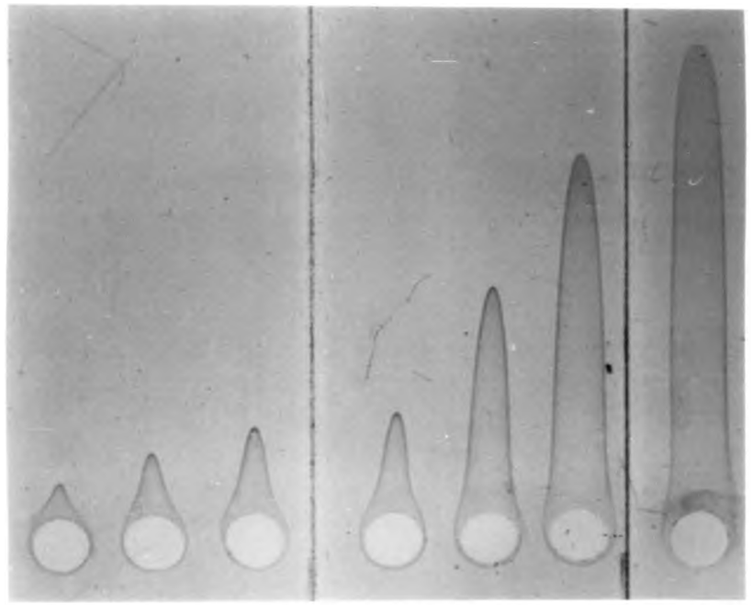


Figure 3.17 - Characterisation by rocket immunoelectrophoresis of purified antiserum raised against RUBISCO large subunit-binding protein complex. Increasing amounts of purified RUBISCO large subunit-binding protein complex were loaded into wells formed in agarose which contained purified serum raised against native RUBISCO large subunit-binding protein complex. Electrophoresis was performed at 90 volts for 16 hours (see Section 2.5.4), after which time the gels were washed with PBS to remove uncomplexed protein, and the immunocomplexes were visualised by Coomassie blue staining.

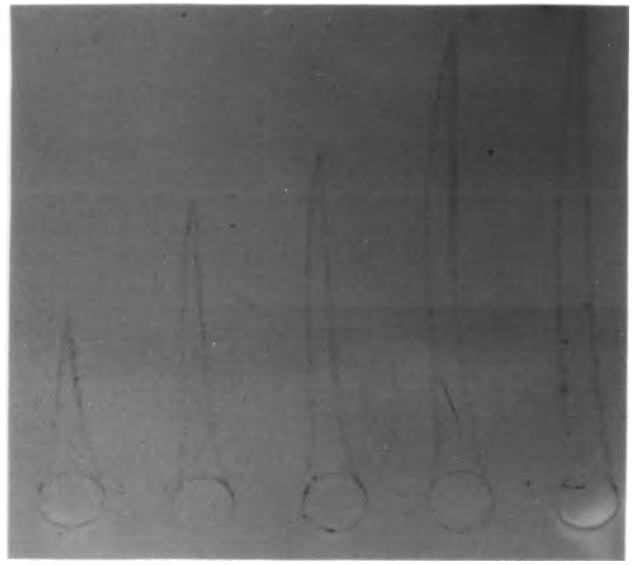


Figure 3.17 - Characterisation by rocket immunoelectrophoresis of purified antiserum raised against RUBISCO large subunit-binding protein complex. Increasing amounts of purified RUBISCO large subunit-binding protein complex were loaded into wells formed in agarose which contained purified serum raised against native RUBISCO large subunit-binding protein complex. Electrophoresis was performed at 90 volts for 16 hours (see Section 2.5.4), after which time the gels were washed with PBS to remove uncomplexed protein, and the immunocomplexes were visualised by Coomassie blue staining.

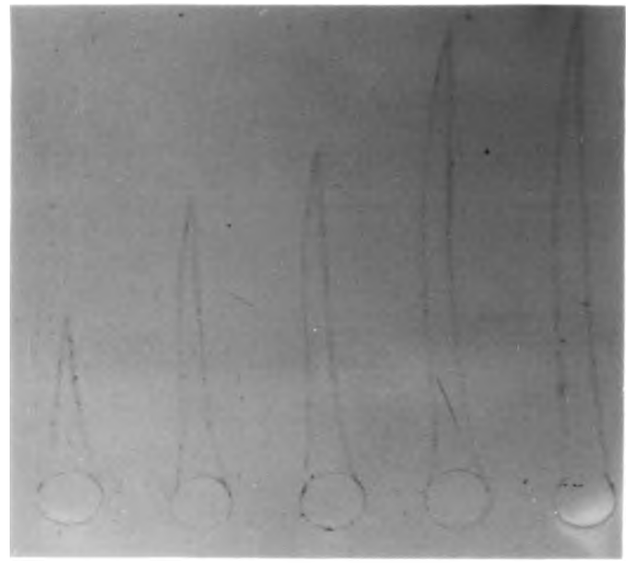


Figure 3.17 - Characterisation by rocket immunoelectrophoresis of purified antiserum raised against RUBISCO large subunit-binding protein complex. Increasing amounts of purified RUBISCO large subunit-binding protein complex were loaded into wells formed in agarose which contained purified serum raised against native RUBISCO large subunit-binding protein complex. Electrophoresis was performed at 90 volts for 16 hours (see Section 2.5.4), after which time the gels were washed with PBS to remove uncomplexed protein, and the immunocomplexes were visualised by Coomassie blue staining.

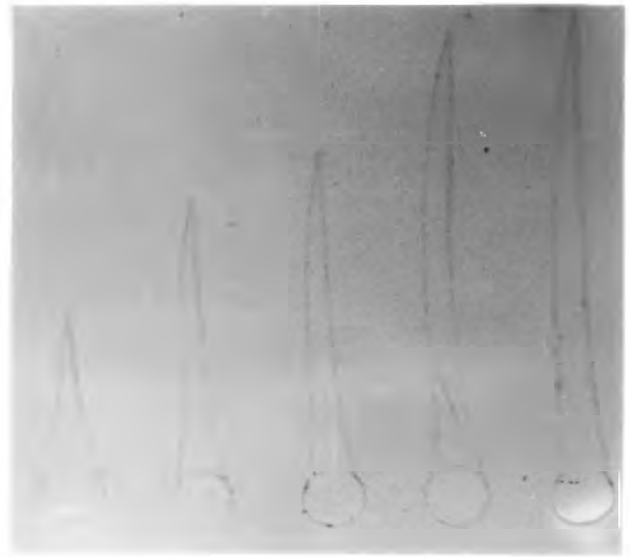


Figure 3.18 - Quantitation of detection of RUBISCO by rocket immunoelectrophoresis. Increasing amounts of RUBISCO were loaded into wells cut in agarose containing RUBISCO antiserum. Electrophoresis was performed overnight, as described in Section 2.5.4) and immunocomplexes were visualised by staining with Coomassie blue. The area under the rockets was determined by projecting at constant magnification onto graph paper and the number of squares under the peak were counted. The area (in arbitrary units) was plotted against µg of RUBISCO loaded. The graph shows the full range standard curve. A standard curve of appropriate scale was prepared for each experiment performed.

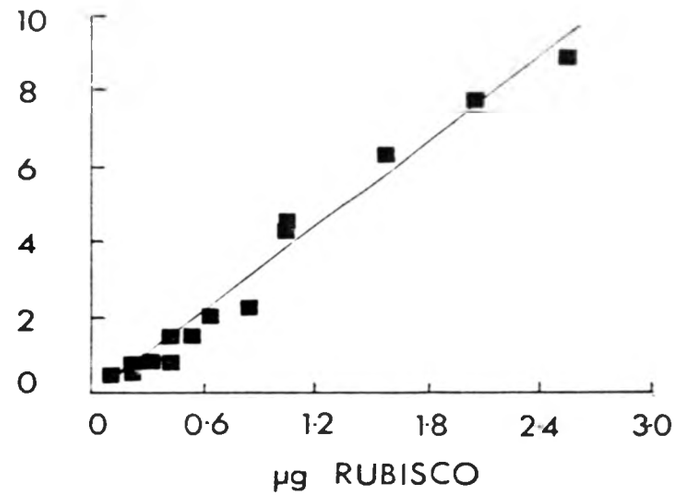
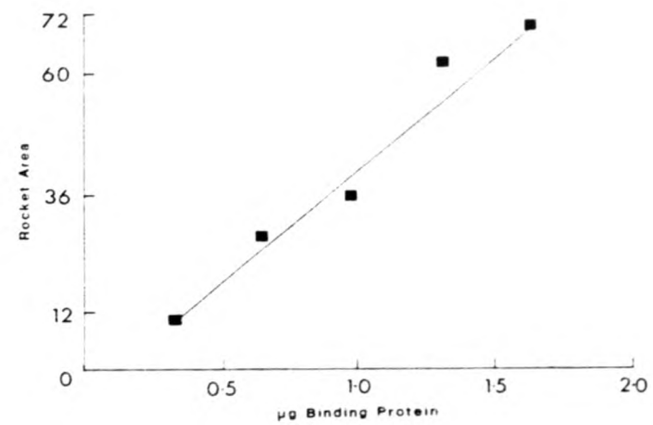


Figure 3.19 – Quantitation of detection of binding protein by rocket immunoelectrophoresis. Increasing amounts of RUBISCO large subunit-binding protein complex were loaded into wells cut in agarose containing RUBISCO large subunit-binding protein antiserum. Electrophoresis was performed overnight and immunocomplexes were visualised by staining with Coomassie blue. The area under the rockets was determined by projecting at constant magnification onto graph paper and the number of squares under the peak were counted. The area (in arbitrary units) was plotted against μg of RUBISCO large subunit-binding protein complex loaded. A standard curve of appropriate scale was prepared for each experiment performed.



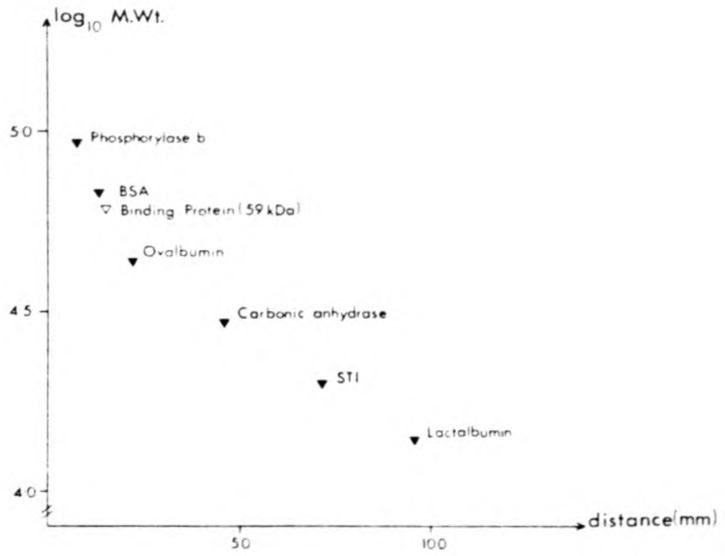
characterisation of the RUBISCO LSU-binding protein complex.

3.2.3 The subunit molecular weight of the LSU-binding protein

Purified RUBISCO LSU-binding protein complex was subjected to electrophoresis under denaturing conditions to allow determination of the molecular weight of the binding protein subunit. The purified complex and molecular weight markers were loaded onto a 15% SDS polyacrylamide gel, and after electrophoresis the proteins were visualised by staining with Coomassie blue. The subunit molecular weight was estimated using the method of Weber & Osborn (1969). The distance moved by the binding protein subunit was compared to those of six molecular weight markers and a semi-logarithmic plot of molecular weight against migration distance prepared (Figure 3.20). The molecular weight of the binding protein subunit was found to be 59,000 by this method. This value is the mean of four determinations and is similar to the value of 60,000 estimated by Barraclough & Ellis (1980). Later work by Hemmingsen & Ellis (1986) shows that if the purified RUBISCO LSU-binding protein complex is subjected to electrophoresis on a 15% SDS polyacrylamide gel with a larger pore size (due to a reduction in the percentage of bisacrylamide present), two types of binding protein subunit can be resolved. These were found to have molecular weights of 61,000 and 60,000. These two binding protein subunits were not resolved with the gel systems used in the course of the

Figure 3.20 - Determination of the molecular weight of the binding protein subunit. The molecular weight was determined by the method of Weber & Osborn (1969). Purified RUBISCO large subunit-binding protein complex was loaded onto a 15% SDS gel (see Section 2.6.1) with molecular weight markers. After electrophoresis proteins were visualised by Coomassie blue staining and the distance moved by the binding protein subunit compared to those of the markers.

The relative molecular masses of the markers are as follows: phosphorylase b - 94,000; BSA - 67,000; ovalbumin - 43,000; carbonic anhydrase - 30,000; STI (soybean trypsin inhibitor) - 20,000; lactalbumin - 14,000.



present work. The two binding protein subunit types have been partially sequenced and have different amino-acids present in the amino-terminal region. There are only two positions in the first 30 residues where the same amino-acids occur. Neither sequence corresponds to that of the RUBISCO LSU (J. E. Musgrove & R. J. Ellis, pers comm.).

Ellis (1977) estimated that the RUBISCO LSU-binding protein complex had a molecular weight in excess of 600,000. This estimation was based on non-denaturing gel electrophoretic studies. More recently, gel filtration studies have yielded a molecular weight estimate of 720,000 (Hemmingsen & Ellis, 1986). Using this latter figure it would appear that the complex is composed of 12 binding protein subunits. The number of RUBISCO large subunits present in the complex is unknown. The large subunits associated with the complex represents a small amount of newly-synthesised, non-assembled large subunits. The complex has been purified with no associated large subunits (Hemmingsen & Ellis, 1986). The stoichiometry of the RUBISCO LSU-binding protein complex has been discussed (Roy et al., 1982). The authors suggest that no more than one large subunit can be bound per complex. If more than four large subunits are associated with the binding protein subunits then there would be a detectable difference in the electrophoretic mobilities of the stainable band and the radioactive LSU on non-denaturing gels. It is possible, however, that some complexes have no large subunits associated, while others possess between one and four large

subunits.

3.2.4 Reversible dissociation of the RUBISCO LSU-binding protein complex

A possible role for the LSU-binding protein complex in the assembly of RUBISCO was proposed by Barraclough & Ellis (1980), when these workers found that newly-synthesised LSU was associated with the binding protein subunit in the complex. The work illustrated in Figures 3.2 and 3.4 indicates that, as newly-synthesised and hence radiolabelled LSU is incorporated into holoenzyme, radioactivity associated with the oligomeric LSU-binding protein complex declines, suggesting but not proving that the complex may function as an intermediate in the assembly of LSU into RUBISCO. The assembly of RUBISCO appears to be light-dependent (Bloom *et al.*, 1983); chloroplasts incubated with radiolabelled amino-acids (to allow synthesis of radiolabelled LSU), which are subsequently incubated in the light during a chase period, exhibit a higher degree of incorporation of labelled LSU into holoenzyme than do similarly treated chloroplasts which have been retained in darkness. This light-driven assembly of RUBISCO appears to rely on ATP. The latter in the presence of Mg^{2+} has been reported to bring about a dissociation of the LSU-binding protein complex (Bloom *et al.*, 1983; Milos & Roy, 1984). This dissociation of the oligomeric form of the binding protein and its associated LSU was investigated further in this work, and a discussion of the

possible importance of this dissociation in relation to the assembly of RUBISCO will be presented below.

It is evident from Figure 3.21 that 10 mM MgATP causes a dissociation of the LSU-binding protein complex, confirming the report of Bloom et al. (1983) However, analysis of the MgATP-treated and untreated chloroplast extracts on 5% non-denaturing gels gives no indication as to the fate of either the binding protein subunits or the small amount of LSU which comprise the oligomeric complex. The smaller oligomers or subunits, which result are not obvious on the gel by staining. Gels with a much greater resolving power are non-denaturing gradient polyacrylamide gels, and these were used in all subsequent studies on the dissociation of the LSU-binding protein complex. On incubation of a stromal extract with increasing concentrations of MgATP, the high molecular weight oligomeric binding protein complex is converted to a faster-migrating form of binding protein as shown in Figures 3.22 and 3.23. The positions of all forms of the binding protein on the non-denaturing gel shown in Figure 3.22 were determined by comparing the stained gel with an identical gel which had been immunoblotted. After "Western" blotting, and incubation of the nitrocellulose filter with anti-binding protein antiserum and ^{125}I-labelled protein A binding protein is detectable in only two positions on the gel (Figure 3.23). The slower-migrating form is the oligomeric LSU-binding protein complex. It is interesting to note that the antiserum reaction with the faster migrating species is stronger than that observed with the oligomeric

Figure 3.21 - The effect of MgATP on the RUBISCO LSU-binding protein complex. A stromal extract was prepared from ten-day old pea plants grown under a 12-hour photoperiod as described in Section 2.1. The extract was incubated at 0°C with 10 mM MgATP for one hour before being subjected to electrophoresis on a 5% non-denaturing gel. After electrophoresis proteins were visualised by staining with Coomassie blue.

Lane markings are as follows: (3) & (4) untreated stromal extract; (1) & (2) stromal extracts treated with MgATP.

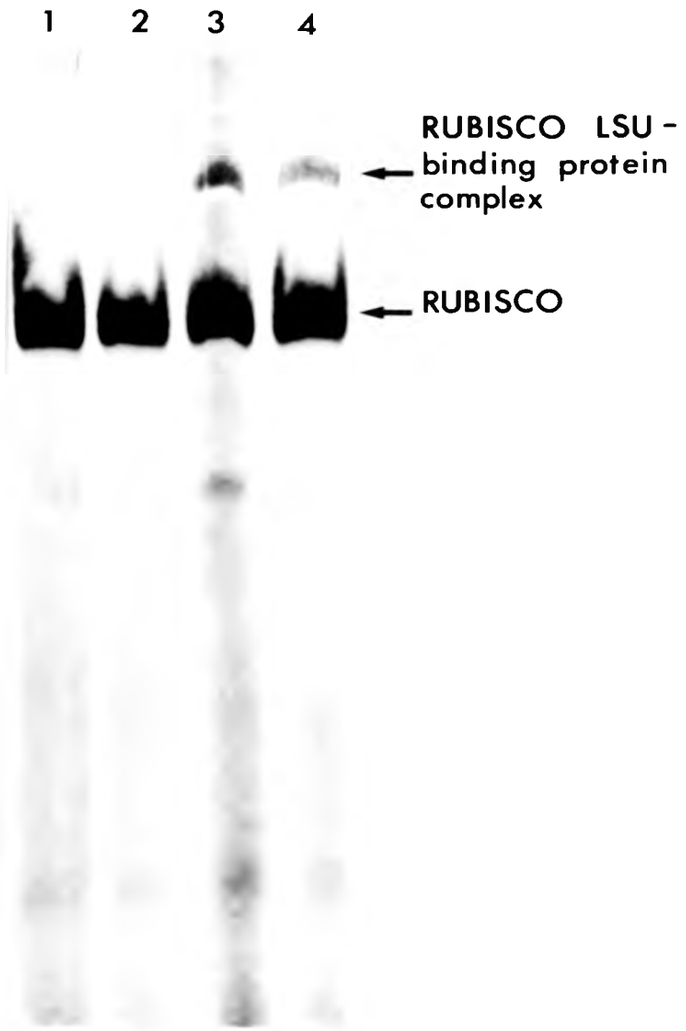


Figure 3.21 - The effect of MgATP on the RUBISCO LSU-binding protein complex. A stromal extract was prepared from ten-day old pea plants grown under a 12-hour photoperiod as described in Section 2.1. The extract was incubated at 0°C with 10 mM MgATP for one hour before being subjected to electrophoresis on a 5% non-denaturing gel. After electrophoresis proteins were visualised by staining with Coomassie blue.

Lane markings are as follows: (3) & (4) untreated stromal extract; (1) & (2) stromal extracts treated with MgATP.

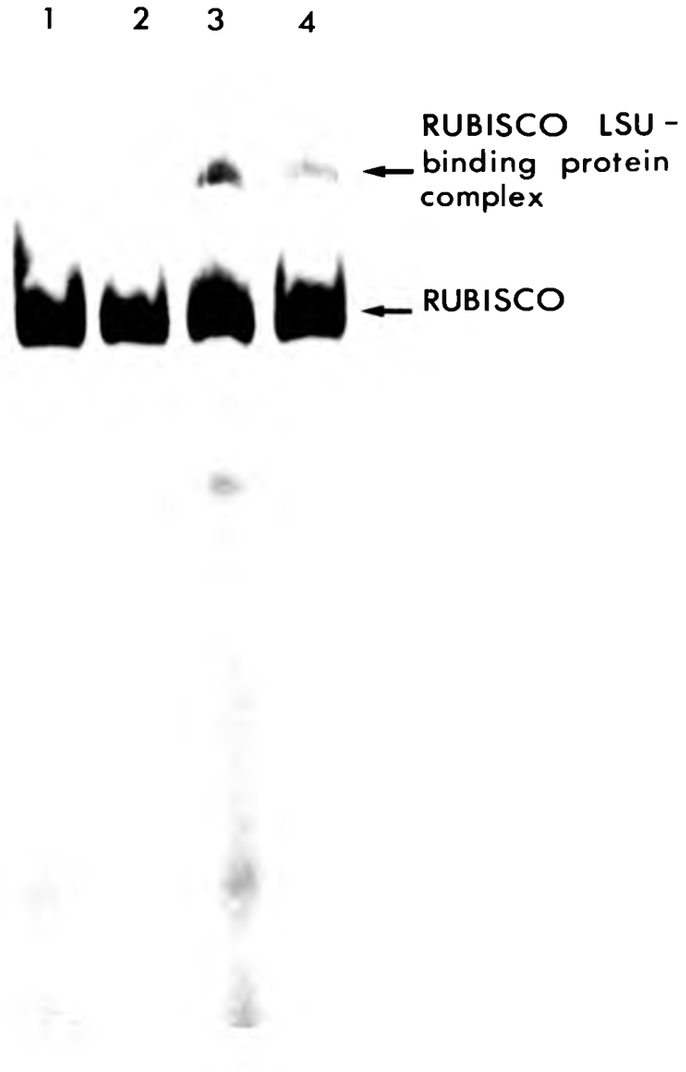


Figure 3.22 - Dissociation of the RUBISCO LSU-binding protein complex by MgATP. A stromal extract was prepared from ten-day old plants grown under a 12-hour photoperiod and aliquots were incubated at 0°C with various concentrations of MgATP for one hour. All aliquots were then analysed by electrophoresis under non-denaturing conditions on a 4%-30% gradient polyacrylamide gel as described in Section 2.6.2.

(A) oligomeric form of binding protein of Mr 720,000; (B) monomeric form of binding protein of Mr 60,000.

The Mr of markers proteins are: carbonic anhydrase - 29,000; albumin, chicken egg - 45,000; albumin, bovine - 66,000 (monomer) & 132,000 (dimer); urease, jack bean - 240,000 (dimer) & 480,000 (tetramer).

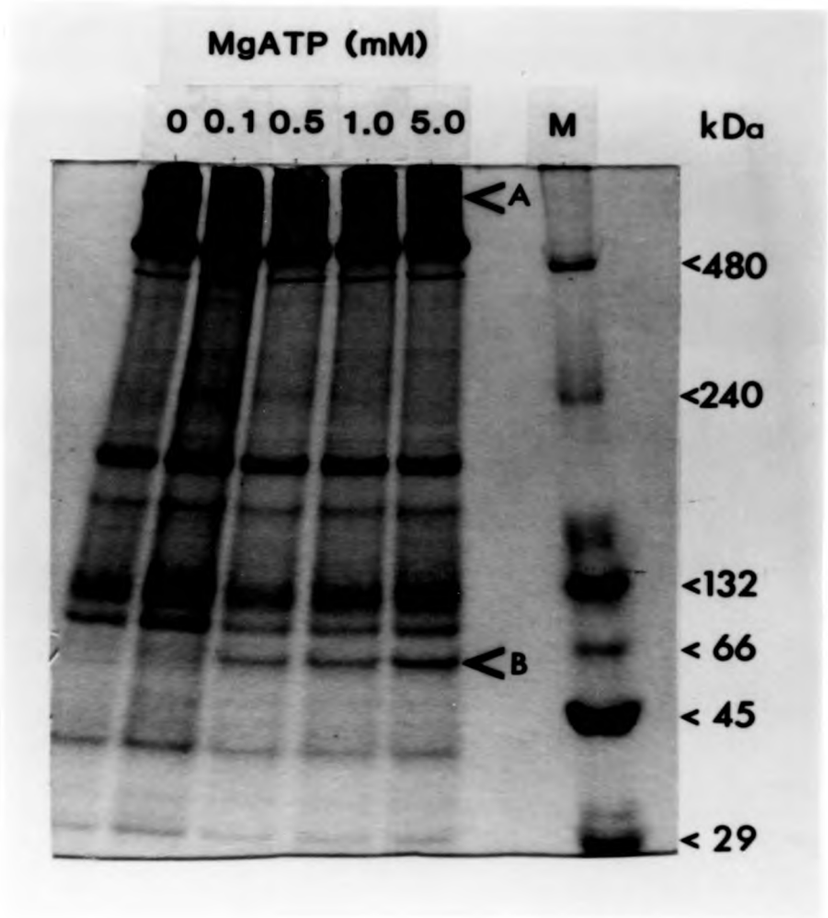


Figure 3.22 - Dissociation of the RUBISCO LSU-binding protein complex by MgATP. A stromal extract was prepared from ten-day old plants grown under a 12-hour photoperiod and aliquots were incubated at 0°C with various concentrations of MgATP for one hour. All aliquots were then analysed by electrophoresis under non-denaturing conditions on a 4%-30% gradient polyacrylamide gel as described in Section 2.6.2.

(A) oligomeric form of binding protein of Mr 720,000; (B) monomeric form of binding protein of Mr 60,000.

The Mr of markers proteins are: carbonic anhydrase - 29,000; albumin, chicken egg - 45,000; albumin, bovine - 66,000 (monomer) & 132,000 (dimer); urease, jack bean - 240,000 (dimer) & 480,000 (tetramer).

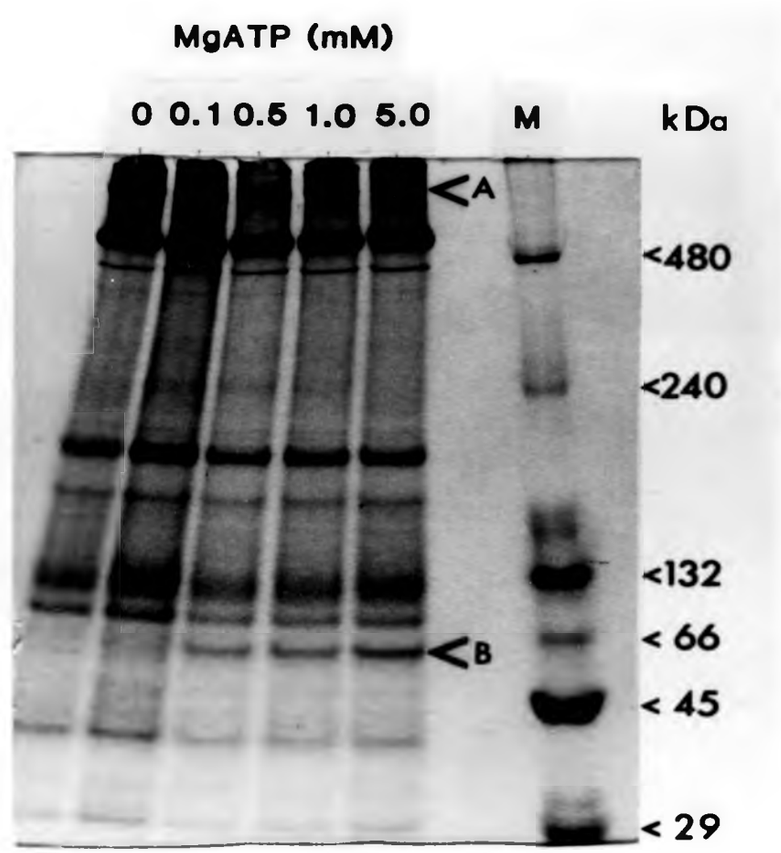


Figure 3.22 - Dissociation of the RUBISCO LSU-binding protein complex by MgATP. A stromal extract was prepared from ten-day old plants grown under a 12-hour photoperiod and aliquots were incubated at 0°C with various concentrations of MgATP for one hour. All aliquots were then analysed by electrophoresis under non-denaturing conditions on a 4%-30% gradient polyacrylamide gel as described in Section 2.6.2.

(A) oligomeric form of binding protein of Mr 720,000; (B) monomeric form of binding protein of Mr 60,000.

The Mr of markers proteins are: carbonic anhydrase - 29,000; albumin, chicken egg - 45,000; albumin, bovine - 66,000 (monomer) & 132,000 (dimer); urease, jack bean - 240,000 (dimer) & 480,000 (tetramer).

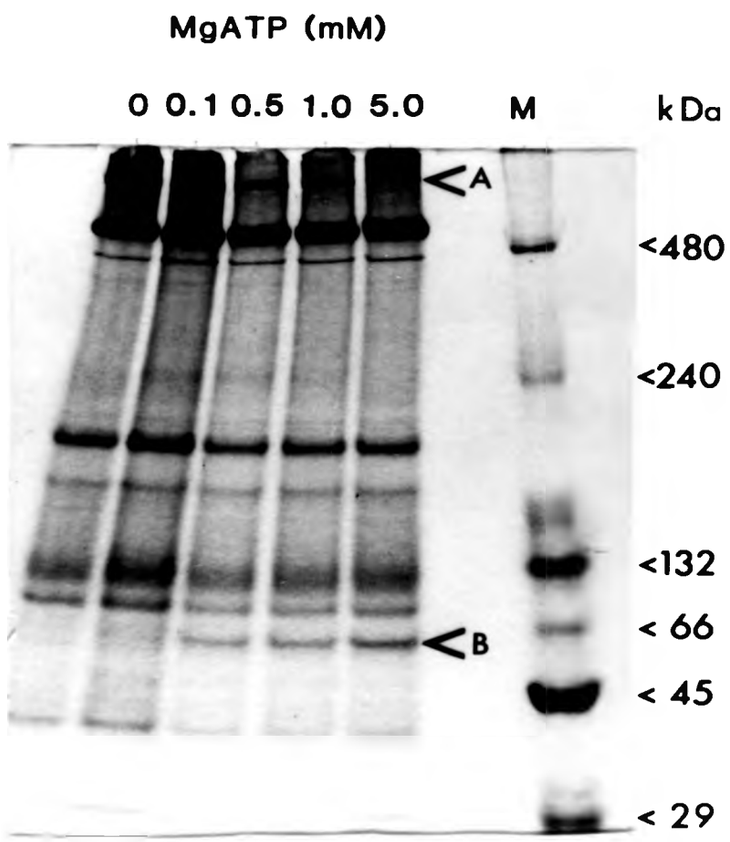
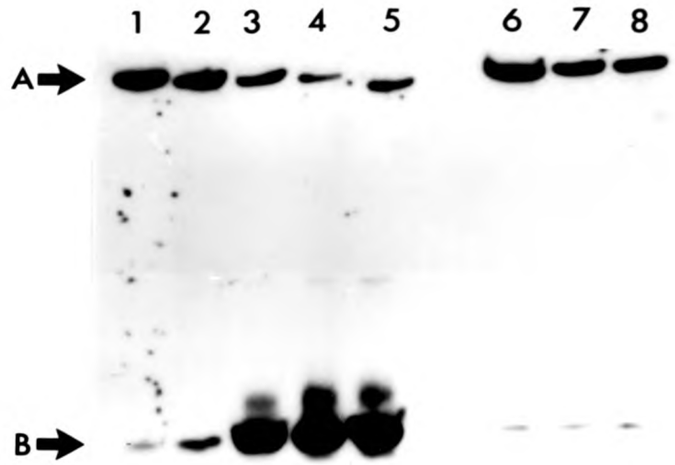


Figure 3.23 - The effect of ATP and GTP on the RUBISCO LSU-binding protein complex. A stromal extract was prepared and dialysed against fresh chloroplast lysis buffer at 4°C for 4 hours. Aliquots of dialysed extract were incubated at 0°C with various concentrations of MgATP or MgGTP for one hour. All aliquots were then subjected to electrophoresis under non-denaturing conditions on a 4%-30% linear gradient gel as described in Section 2.6.2. The proteins were blotted onto nitrocellulose and the positions of binding protein were determined by incubating the filter with anti-binding protein antiserum and ^{125}I-labelled protein A. The figure shows the autoradiograph of the nitrocellulose filter.
The experiment was performed with assistance from Mrs. J.E. Musgrove.

Lane markings are as follows: (1) no ATP; (2) 0.1 mM ATP; (3) 0.5 mM ATP; (4) 1 mM ATP; (5) 5 mM ATP; (6) 0.5 mM GTP; (7) 1 mM GTP; (8) 5 mM GTP. A - binding protein oligomer of Mr 720,000; B - binding protein monomer of Mr 60,000.



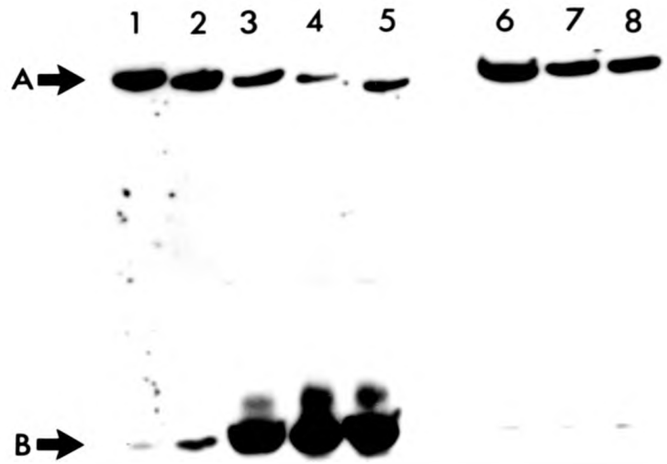


Figure 3.23 - The effect of ATP and GTP on the RUBISCO LSU-binding protein complex. A stromal extract was prepared and dialysed against fresh chloroplast lysis buffer at 4°C for 4 hours. Aliquots of dialysed extract were incubated at 0°C with various concentrations of MgATP or MgGTP for one hour. All aliquots were then subjected to electrophoresis under non-denaturing conditions on a 4%-30% linear gradient gel as described in Section 2.6.2. The proteins were blotted onto nitrocellulose and the positions of binding protein were determined by incubating the filter with anti-binding protein antiserum and ^{125}I-labelled protein A. The figure shows the autoradiograph of the nitrocellulose filter.
The experiment was performed with assistance from Mrs. J.E. Musgrove.

Lane markings are as follows: (1) no ATP; (2) 0.1 mM ATP; (3) 0.5 mM ATP; (4) 1 mM ATP; (5) 5 mM ATP; (6) 0.5 mM GTP; (7) 1 mM GTP; (8) 5 mM GTP. A - binding protein oligomer of Mr 720,000; B - binding protein monomer of Mr 60,000.

complex. It may be that antigenic sites masked in the latter become exposed on dissociation of the complex.

An estimation of the molecular weight of the faster migrating form of the binding protein shown in Figures 3.22 and 3.23 was obtained by comparing the migration distance of the latter with those of molecular weight markers. Attention has been drawn to the importance of both size and charge in the separation of proteins by non-denaturing polyacrylamide gel electrophoresis (Hedrick & Smith, 1968). Determination of the molecular weights of native proteins must involve the preparation of a Ferguson plot (a plot of gel concentration against log of relative mobility), the slope of which yields the molecular weight. Such a technique is time-consuming. However, it has been shown that it is impossible for a native protein to migrate to a position on a non-denaturing gel which would indicate a lower molecular weight than authentic (Firgaira et al., 1981). Therefore, it is clear that the faster migrating form of binding protein shown in Figures 3.22 and 3.23 is the monomeric form of binding protein with a molecular weight of 60,000.

A small proportion of the monomeric form of binding protein is present even in chloroplast extracts which have been dialysed to remove endogenous MgATP (Figure 3.23; lane 1). Levels of MgATP as low as 0.1 mM have some effect on dissociation; however some of the oligomeric form is still present at 5 mM MgATP. GTP in the presence of Mg^{2+} has no effect on the oligomeric complex (Figure 3.23), a finding which is in agreement with the work of Bloom et al. (1983).

These workers also reported that a non-hydrolysable analogue of ATP was without effect on the complex, and proposed that ATPase activity is required to cause a dissociation of the oligomeric complex. Hemmingsen & Ellis (1986) have shown that the binding protein subunits do not appear to be either phosphorylated or adenylated on treatment with ATP.

The fate of the dissociated binding protein subunits in chloroplast extracts was further analysed by sucrose density gradient centrifugation. The profile of proteins in gradients both in the presence and absence of MgATP was revealed by immunoblotting (Figures 3.24 and 3.25). Some low molecular weight form of the binding protein was present in the top-most fractions in the absence of MgATP, consistent with the findings shown in Figure 3.23. However, the bulk of the binding protein was present in the oligomeric LSU-binding protein form (Figure 3.24; fraction numbers 10 and 12). Measurement of the distribution of radiolabelled proteins across the nitrocellulose filter in the absence of MgATP is shown in Figure 3.26. In the presence of 10 mM MgATP there is a dissociation of the oligomeric form, and a resultant increase in antibody reaction against binding protein present at the top of the gradient, as shown in Figure 3.25. Figure 3.27 shows this shift in the position of binding protein in quantitative terms.

Removal of added ATP by allowing protein synthesis to proceed in a chloroplast extract permits a reformation of the oligomeric form of binding protein (Figure 3.28). This figure shows that at zero time, and in the presence of 2 mM ATP,

Figure 3.24 - Analysis of stromal proteins by sucrose density gradient centrifugation. Stromal proteins were extracted from pea plants grown under a 12-hour photoperiod and were loaded onto a 5%-50% (w/v) sucrose step gradient as described in Section 2.7.4. After centrifugation the gradient was fractionated and selected fractions were subjected to electrophoresis under denaturing conditions. The proteins were transferred onto a nitrocellulose filter by "Western" blotting and the positions of binding protein, LSU and SSU determined by incubating the filter with antiserum raised against the proteins and ^{125}I-labelled protein A. The filter was exposed to X-ray film for 18 hours. Markers shown (M) are (a) RUBISCO LSU-binding protein subunit; (b) RUBISCO LSU; (c) RUBISCO SSU.

The top and bottom of the gradient are marked. Equal volumes of each fraction were loaded.

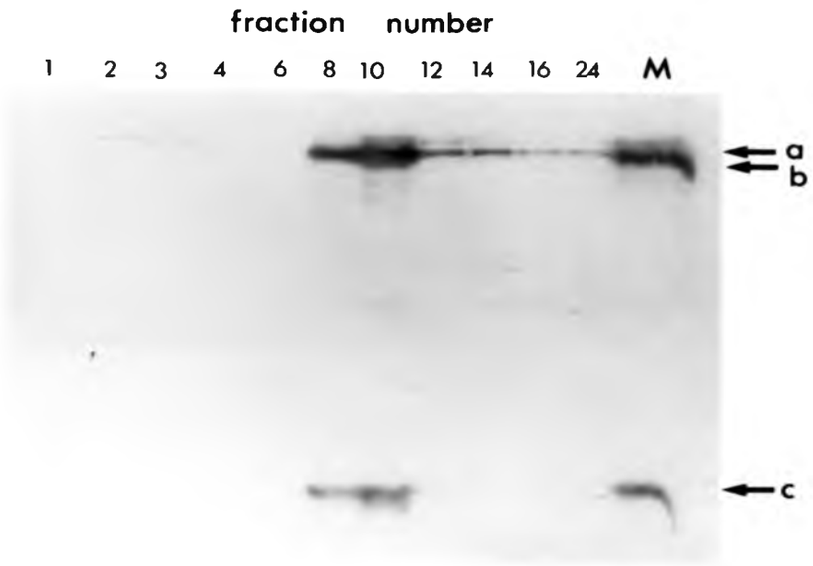


Figure 3.24 – Analysis of stromal proteins by sucrose density gradient centrifugation. Stromal proteins were extracted from pea plants grown under a 12-hour photoperiod and were loaded onto a 5%–50% (w/v) sucrose step gradient as described in Section 2.7.4. After centrifugation the gradient was fractionated and selected fractions were subjected to electrophoresis under denaturing conditions. The proteins were transferred onto a nitrocellulose filter by "Western" blotting and the positions of binding protein, LSU and SSU determined by incubating the filter with antiserum raised against the proteins and ^{125}I-labelled protein A. The filter was exposed to X-ray film for 18 hours. Markers shown (M) are (a) RUBISCO LSU-binding protein subunit; (b) RUBISCO LSU; (c) RUBISCO SSU.

The top and bottom of the gradient are marked. Equal volumes of each fraction were loaded

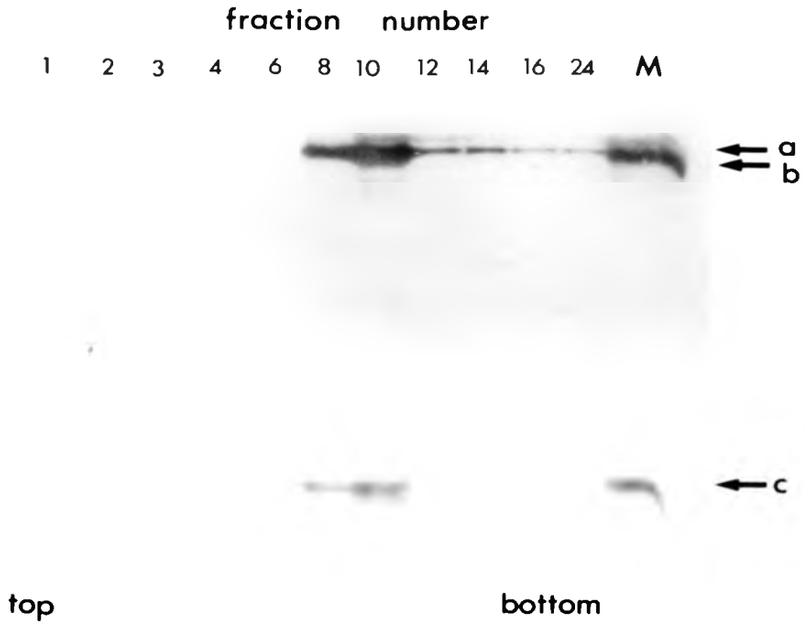


Figure 3.25 - Analysis of stromal proteins by sucrose density centrifugation in the presence of ATP. Stromal proteins were extracted from pea plants grown under a 12-hour photoperiod and were incubated in the presence of 10 mM MgATP for one hour at 0°C before centrifugation on a 5%-50% (w|v) sucrose step gradient containing 10 mM MgATP as described in Section 2.7.4. After centrifugation the gradient was fractionated and selected fractions were subjected to electrophoresis under denaturing conditions. The proteins were transferred onto a nitrocellulose filter by "Western" blotting and the positions of binding protein, LSU and SSU determined by incubating the filter with antiserum raised against the proteins and ^{125}I-labelled protein A. The filter was exposed to X-ray film for 18 hours. Markers shown (M) are (a) RUBISCO LSU-binding protein subunit; (b) RUBISCO LSU; (c) RUBISCO SSU.
The top and bottom of the gradient are marked. Equal volumes of each fraction were loaded.

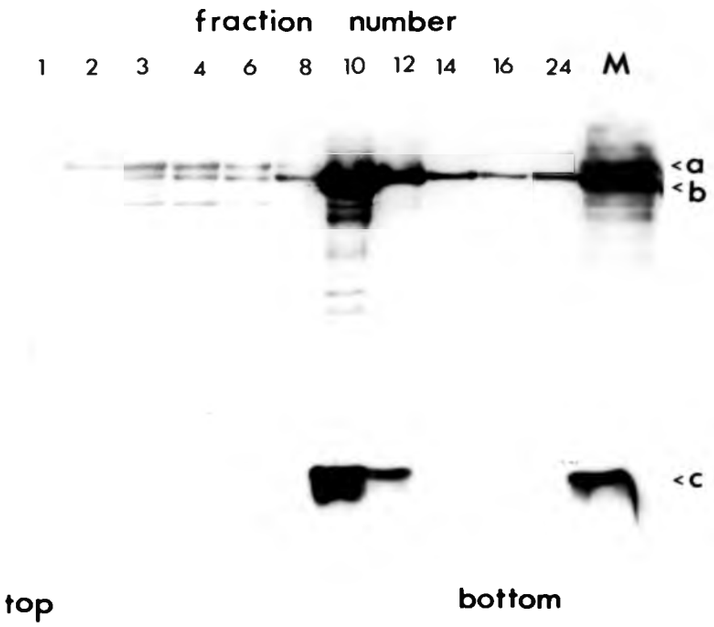


Figure 3.25 - Analysis of stromal proteins by sucrose density centrifugation in the presence of ATP. Stromal proteins were extracted from pea plants grown under a 12-hour photoperiod and were incubated in the presence of 10 mM MgATP for one hour at 0°C before centrifugation on a 5%-50% (w|v) sucrose step gradient containing 10 mM MgATP as described in Section 2.7.4. After centrifugation the gradient was fractionated and selected fractions were subjected to electrophoresis under denaturing conditions. The proteins were transferred onto a nitrocellulose filter by "Western" blotting and the positions of binding protein, LSU and SSU determined by incubating the filter with antiserum raised against the proteins and ^{125}I-labelled protein A. The filter was exposed to X-ray film for 18 hours. Markers shown (M) are (a) RUBISCO LSU-binding protein subunit; (b) RUBISCO LSU; (c) RUBISCO SSU.
The top and bottom of the gradient are marked. Equal volumes of each fraction were loaded.

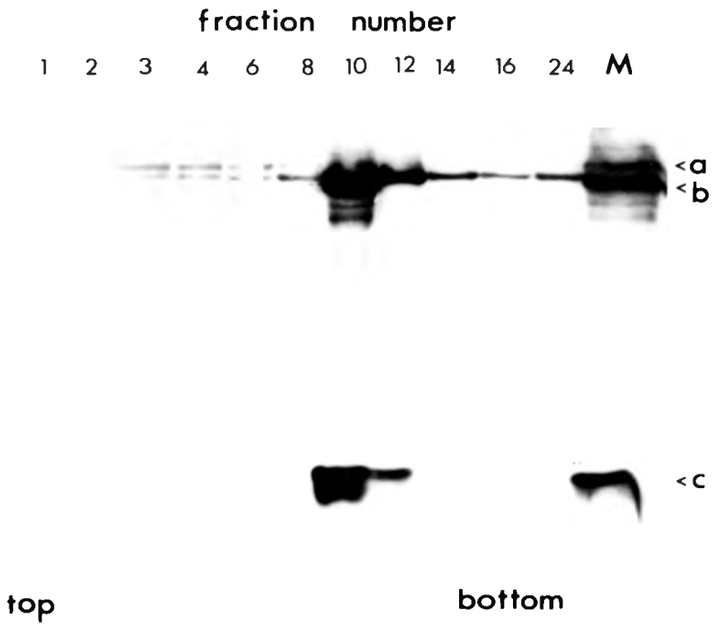


Figure 3.26 - Centrifugation profile of RUBISCO subunits and the RUBISCO LSU-binding protein. The positions of LSU, SSU and binding protein subunits were determined by excising pieces of nitrocellulose filter from the blot shown in Figure 3.24 and counting for radioactivity as described in Section 2.5.3. All counts were corrected for background, which was determined by counting pieces of filter which gave no darkening of the X-ray film. (A) LSU; (B) SSU; (C) RUBISCO LSU-binding protein.

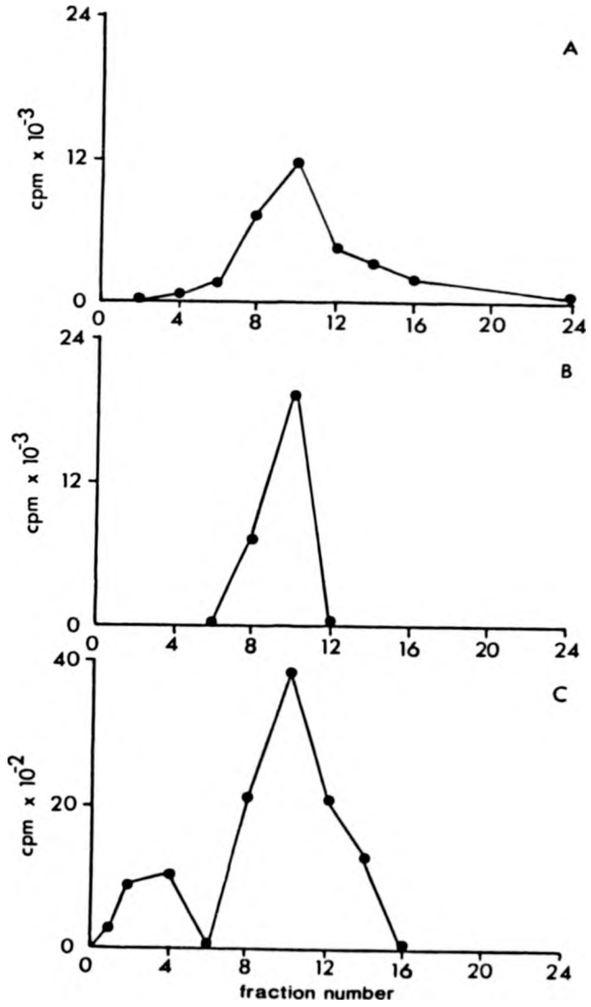


Figure 3.27 - Centrifugation profile of RUBISCO subunits and the RUBISCO LSU-binding protein in the presence of ATP. The positions of LSU, SSU and binding protein subunits were determined by excising pieces of nitrocellulose filter from the immunoblot shown in Figure 3.25 and counting for radioactivity. All counts were corrected for background, which was determined by counting pieces of filter which gave no darkening of the X-ray film. (A) LSU; (B) SSU; (C) RUBISCO LSU-binding protein.

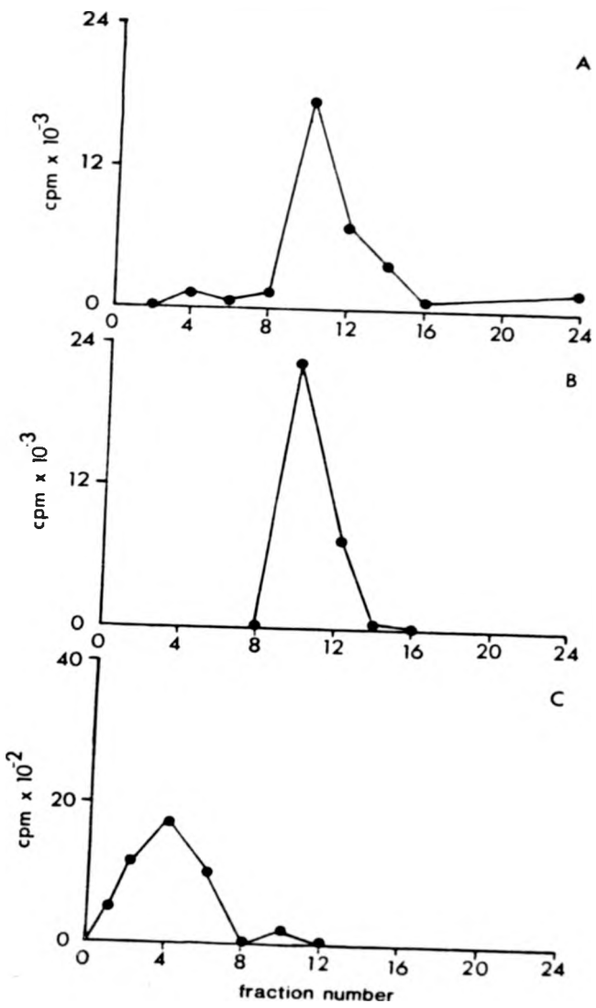


Figure 3.28 - Reversible dissociation of the RUBISCO LSU-binding protein complex. Chloroplasts isolated from eight-day old pea plants grown under a 12-hour photoperiod were lysed in a solution containing 25 mM Tris-HCl (pH 8.0) and 10 mM $MgSO_4$. An aliquot was removed and maintained at 0°C as the zero time control. The remainder of the stromal extract was then incubated at 25°C under conditions suitable for protein synthesis by chloroplast ribosomes in a medium containing 2 mM ATP; 0.2 mM GTP and 80 mM KCl (see Section 2.3.3). At intervals aliquots were removed and these were subjected to electrophoresis on 4%-30% gradient non-denaturing gels and the proteins were subsequently transferred onto a nitrocellulose filter. Positions of the RUBISCO LSU-binding protein were determined by incubation of the filter with antiserum raised against the RUBISCO LSU-binding protein and ^{125}I-labelled protein A. Molecular weights were estimated by comparison with molecular weight markers. (A) binding protein oligomer, Mr = 720,000; (B) binding protein monomer, Mr = 60,000. The experiment was performed with assistance from Mrs. J.E. Musgrove.

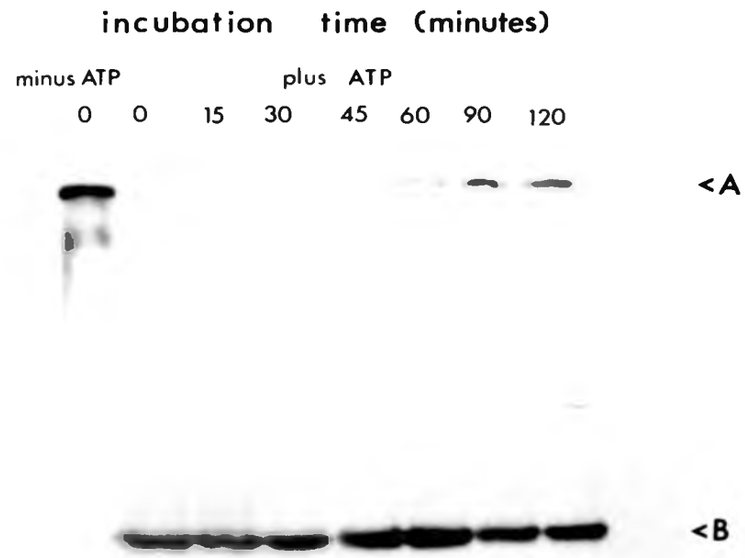
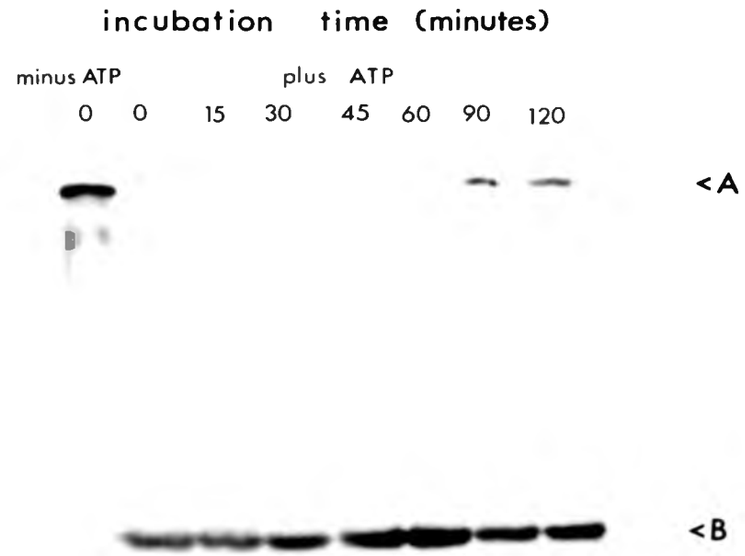


Figure 3.28 - Reversible dissociation of the RUBISCO LSU-binding protein complex. Chloroplasts isolated from eight-day old pea plants grown under a 12-hour photoperiod were lysed in a solution containing 25 mM Tris-HCl (pH 8.0) and 10 mM $MgSO_4$. An aliquot was removed and maintained at 0°C as the zero time control. The remainder of the stromal extract was then incubated at 25°C under conditions suitable for protein synthesis by chloroplast ribosomes in a medium containing 2 mM ATP; 0.2 mM GTP and 80 mM KCl (see Section 2.3.3). At intervals aliquots were removed and these were subjected to electrophoresis on 4%-30% gradient non-denaturing gels and the proteins were subsequently transferred onto a nitrocellulose filter. Positions of the RUBISCO LSU-binding protein were determined by incubation of the filter with antiserum raised against the RUBISCO LSU-binding protein and ^{125}I-labelled protein A. Molecular weights were estimated by comparison with molecular weight markers. (A) binding protein oligomer, Mr = 720,000; (B) binding protein monomer, Mr = 60,000. The experiment was performed with assistance from Mrs. J.E. Musgrove.



there is none of the oligomeric form of the binding protein present at all. In this latter experiment, ATP is utilised by protein synthesis on chloroplast ribosomes and as this proceeds, a reappearance of the oligomeric form is obvious. There is also a concurrent decline in the intensity of the immunoreaction with the monomeric form of binding protein. A point of considerable interest is whether or not ATP is formed on this reappearance. No information is available concerning this at present.

3.2.5 DISCUSSION

The concentration of ATP in the chloroplast stroma *in vivo* is greater than 0.5 mM and concentrations rise on illumination to greater than 1 mM (Hampp *et al.*, 1982). Therefore the dissociation/reassociation reactions presented in Figures 3.22 - 3.28 occur within the physiological range of ATP concentration. The presence of some binding protein monomer in the complete absence of ATP suggests that an equilibrium exists between the two forms of binding protein. The position of this equilibrium would appear to be determined by the ATP concentration. The equilibrium proposed by Lennox & Ellis (1985) can be represented as follows:

$$\alpha 6\beta 6 \rightleftharpoons 6\alpha + 6\beta$$

where α and β refer to the two types of binding protein subunit which exist. It is clear that under physiological conditions, at levels of between 1 mM and 2 mM ATP, the bulk of the binding protein will be present in the chloroplast in

the monomeric form, although a proportion of the protein in the oligomeric form will exist. However, on preparation of a chloroplast extract, the stromal components, including ATP, will be diluted by a factor of at least 100-fold. This will result in a shift of the above equilibrium to the left, with the recruitment of most of the binding protein monomers into the oligomeric complex which is visible as a staining band on non-denaturing gels and which can be easily purified.

The fate of newly-synthesised LSU on dissociation of the oligomeric form of the binding protein remains to be elucidated. The estimated molecular weight of 60,000 for the dissociated binding protein would suggest that the newly-synthesised LSUs do not remain associated with the binding protein monomers. In vivo, however, as the ATP concentration is raised, there may be an association of the released LSU with free SSU, resulting in RUBISCO assembly. In support of the model that the binding protein is involved in RUBISCO assembly, ATP-released LSU have been proposed to participate in the assembly of RUBISCO in chloroplasts which have been subjected to a pulse-chase treatment and then incubated with 5 mM MgATP, to facilitate dissociation of the oligomer (Milos & Roy, 1984). Attempts to repeat these observations at Warwick have so far been unsuccessful. Milos & Roy (1984) suggest that the complex may act as an obligatory intermediate in RUBISCO assembly, but this hypothesis awaits confirmation. It has been reported that anti-binding protein antiserum inhibit the assembly of newly-synthesised LSU into RUBISCO (H. Roy, pers. comm.), a second line of evidence consistent

with the above theory.

A precedent does exist for the involvement of a protein in the assembly of another oligomeric protein. This precedent is the protein nucleoplasmin, whose presence is essential for the correct formation of nucleosomes from separated DNA and histones (Laskey et al., 1978). Nucleoplasmin binds to histones and transfers these to DNA.

A second proposed hypothesis as to the function of the binding protein complex is that it may act as storage site for unassembled LSU which cannot immediately assemble into holoenzyme because, for example of a lack of SSU (Milos & Roy, 1984). Release of these LSU by MgATP would allow assembly. This is also an attractive proposal, as LSU are notoriously insoluble in aqueous media when removed from the holoenzyme. LSU synthesised from cloned genes in E. coli are also present in an insoluble form, suggesting that even in vivo solubility is a problem (Gatenby, 1984). These and other possible roles for the binding protein will be discussed more fully in Section 4.

3.3 PHOTOREGULATION OF THE LSU-BINDING PROTEIN AND RUBISCO

3.3.1 Light-stimulated accumulation of RUBISCO and the LSU-binding protein

A large proportion of the literature which has been published regarding the synthesis of RUBISCO concerns its

regulation by light. In some species, most notably the cereals, RUBISCO is easily detectable in dark-grown plants (Tobin & Silverthorne, 1985; Kobayashi et al., 1980), while in others there is a marked increase in the amount of RUBISCO on illumination (Jenkins et al., 1983; Freyssinet et al., 1984). In such species the increase in the amount of protein occurs in parallel with the increase in the capacity of the tissue to fix CO_2 (Graham et al., 1968). It was of interest, therefore, to investigate the role of light in the control of the synthesis of the LSU-binding protein to determine whether the control paralleled that of either of the subunits of RUBISCO.

Two experimental systems were used in this study. The first experimental system employed was one in which etiolated pea apices were exposed to white light for increasing periods of time. Much of our existing knowledge concerning the development of the chloroplast has come from the study of the changes which accompany the differentiation of the etioplast into chloroplast in just such a system (Kirk & Tilney-Bassett, 1978; Sasaki et al., 1981; Jenkins et al., 1983). The use of such a system has been criticised on the grounds that under normal growth conditions full development of proplastid into etioplast does not occur, and hence that differentiation of the latter into a chloroplast may not represent the typical pathway in chloroplast development (Leech et al., 1973). However, the light-regime known as "greening" is convenient to use, and serves as a model system in which development and expression of specific

genes can be studied. It has been stated that the control mechanisms presumably involved in chloroplast biosynthesis are the same in such a regime as occur naturally (Kirk & Tilney-Bassett, 1978), and it was therefore felt that use of such a light-regime was justified.

Light effects gross morphological changes on etiolated P. sativum plants, most notably the rate of stem elongation decreases markedly and the leaves of the apical bud begin to expand (Ellis et al., 1984). Some of the biochemical changes brought about on plant illumination are shown in Figures 3.29 and 3.30. Dark-grown plants are devoid of chlorophyll but on illumination, after an initial lag period, there is a rapid accumulation of chlorophyll (Figure 3.30). Chlorophyll is formed from protochlorophyllide which accumulates in dark-grown plants. This protochlorophyllide is converted to chlorophyll in a light-dependent reaction which is catalysed by the enzyme NADPH-protochlorophyllide-oxidoreductase (Bradbeer, 1981).

Fresh weight and total soluble protein increase five-fold over the 48-hour illumination period (Figure 3.29). These increases are consistent with the findings of Ellis et al., (1984), who found that after an identical illumination period the fresh weight of the apical buds had quadrupled while DNA content had tripled.

The rates of accumulation of RUBISCO and the LSU-binding protein are shown in Figure 3.31. RUBISCO is present in low amounts in the dark-grown plants, but on illumination and after a lag of 24 hours, there is a rapid

Figure 3.29 - Increases in fresh weight and soluble protein on illumination of etiolated Pisum sativum plants. Apical buds from etiolated plants, and etiolated plants subjected to increasing periods of illumination, were excised, weighed and total soluble protein extracted as described in Section 2.2. Protein amounts were determined by the dye-binding method of Bradford (1976). Symbols in brackets represent amounts of soluble protein and fresh weights of apical buds of plants retained in total darkness for 48 hours.

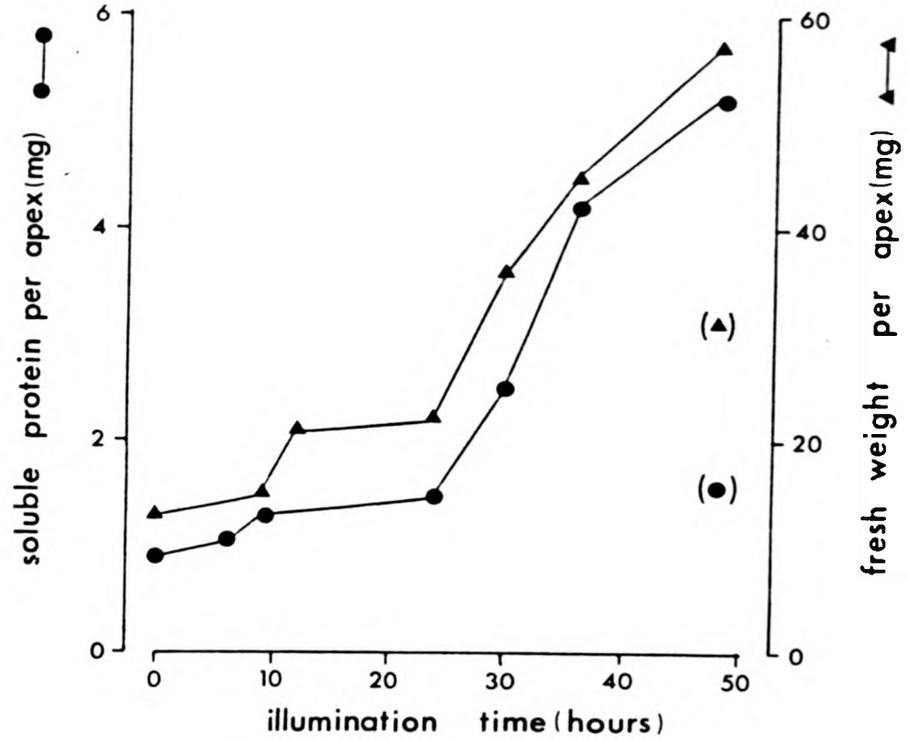
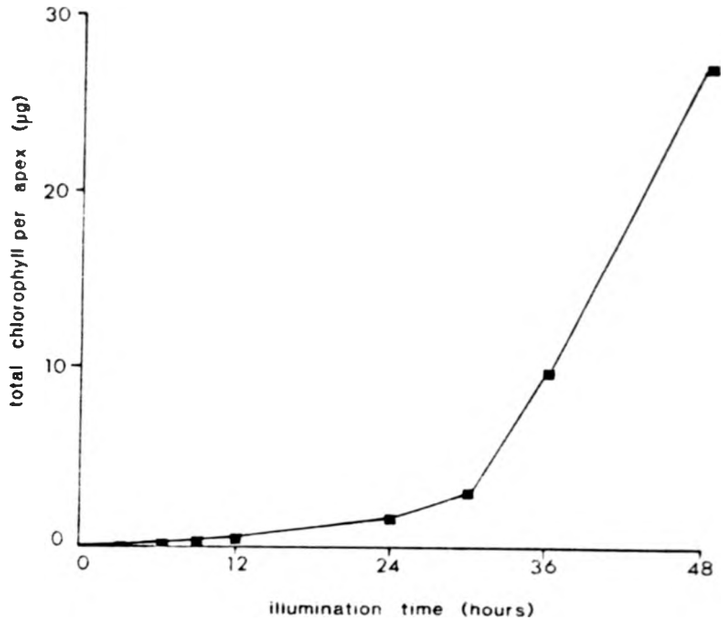


Figure 3.30 - Accumulation of chlorophyll on illumination of etiolated plants of Pisum sativum. Total chlorophyll was extracted from apical buds which were either grown in complete darkness or had been grown in complete darkness and then subjected to increasing periods of illumination, as described in Section 2.1

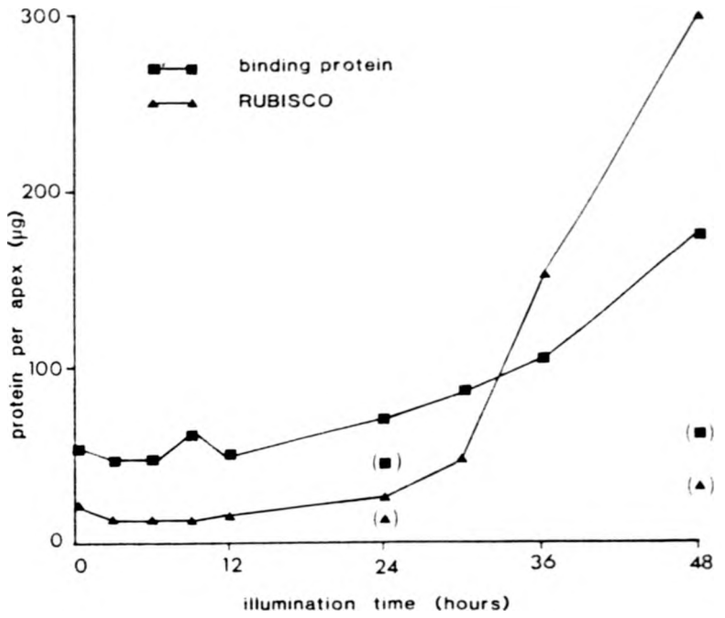


accumulation of RUBISCO. The increase over the 48-hour period is thirty-fold. RUBISCO levels expressed as a percent of total soluble protein reveals the extent of the induction of the holoenzyme by light. In etiolated plants RUBISCO accounts for 2% of the total soluble protein, while in etiolated plants which have been exposed to 48 hours of light the RUBISCO accounts for 13% of the total soluble protein. Similar values were reported by Sasaki et al. (1981).

RUBISCO LSU-binding protein similarly can be detected in dark-grown apical buds (Figure 3.31). The finding that RUBISCO does not accumulate to any extent in etiolated plants is therefore not the result of LSU insolubility due to a lack of binding protein subunits. Amounts of binding protein increase steadily following the illumination of etiolated plants although this increase is only three or four-fold. This increase is similar to that seen in DNA amounts on illumination (Ellis et al., 1984). Thus it appears that the amount of binding protein per cell does not increase as a result of illumination, and that light may have no affect on the expression of the nuclear genes which encode the binding protein subunits. However, it is possible that both the rates of synthesis and breakdown of the binding protein are stimulated by light, which would result in a constant level of binding protein in the cell. The binding protein does not therefore appear to be photoregulated in the same manner as does RUBISCO, although its accumulation is stimulated by light.

The second experimental system used to study the

Figure 3.31 - Light-stimulated accumulation of RUBISCO and RUBISCO LSU-binding protein. Apical buds were excised from plants which had been grown in total darkness and from etiolated plants subjected to increasing periods of illumination. Total soluble protein was extracted and amounts of RUBISCO and RUBISCO LSU-binding protein determined by rocket immunoelectrophoresis as described in Section 2.5.4. Symbols in brackets refer to extracts from plants maintained in darkness for 24 and 48 hours.



light-stimulated accumulation of RUBISCO and the LSU-binding protein was one in which Pisum plants raised from seed in total darkness were compared with plants raised under a 12-hour photoperiod for the same period of time. Again a number of parameters were measured and the results, which are presented in Table 3.1, allow a direct comparison to be made on the effect of light on the development of Pisum plants, and on the accumulation of RUBISCO and the LSU-binding protein. Light effects a 5-fold increase in both the fresh weight and soluble protein contents of the apical buds. An increase of similar magnitude is observed for the LSU-binding protein (7-fold), consistent with the findings shown in Figure 3.31. The increase observed for RUBISCO is much greater (30-fold). Clearly light is much more important in the control of RUBISCO subunit expression and this aspect of the regulation of RUBISCO synthesis was further studied in the next section.

3.3.2 Synthesis of the subunits of RUBISCO on illumination of etiolated plants

Much of the information regarding the photoregulation of the RUBISCO subunits has been gained from studies on the control of amounts of mRNAs for both LSU and SSU (Bennett et al., 1984; Sasaki et al., 1983; Tobin & Silverthorne, 1985). The following work, which examines photocontrol of LSU and SSU and the LSU-binding protein at the protein level, complements the earlier work and will be

Table 3.1 - Comparison of RUBISCO and RUBISCO LSU-binding protein contents in light-grown and dark-grown pea plants. Pea seeds were germinated either in complete darkness at 20°C or under a 12-hour photoperiod at 20°C for 8 days. Apical buds were excised, weighed and total soluble protein extracted and estimated as described in Section 2.2. The amounts of RUBISCO and RUBISCO LSU-binding protein present in the extracts were determined by rocket immunoelectrophoresis.

	Amount per apical bud (mg)			
Growth condition	Fresh weight	Soluble protein	RUBISCO	RUBISCO LSU - binding protein
Darkness	22	0.8	0.11	0.014
light	108	4.0	3.32	0.097

discussed in relation to this work.

Initially, the protein profile from plants which had been exposed to increasing periods of illumination was examined using the highly sensitive method of protein detection involving silver staining. This technique has been used to detect proteins at the nanogram level (Wray et al., 1981). The protein profile resulting from loading one twentieth of one apical bud is shown in Figure 3.32. It is clear that on illumination there is an accumulation of a large number of polypeptides including LSU and SSU. The LSU of RUBISCO is present in etiolated plants, consistent with the finding of Siddell & Ellis (1975). The striking finding is that there appears to be no SSU present in dark-grown plants and, indeed, the polypeptide is not visible until the plants have been exposed to 24 hours of illumination. The more sensitive technique of "Western" blotting was employed to provide a second line of evidence for this finding and the result is shown in Figure 3.33. Again, if protein equivalent to one twentieth of one apical bud is loaded onto a polyacrylamide gel, and immunoblotted, no SSU is evident until the late stages of illumination. Both LSU and the LSU-binding protein are present in etiolated plants, with amounts increasing throughout the 48-hour illumination period. The increase in LSU as detected by antiserum appears to be greater than that observed for the binding protein, as expected from Figure 3.31.

To determine whether this absence of SSU was in fact

Figure 3.32 - Accumulation of RUBISCO subunits on illumination of pea plants as determined by silver staining. Apical buds were excised from etiolated pea plants, and from etiolated plants which had been subjected to illumination for increasing periods of time. Total soluble protein was extracted and protein equivalent to one twentieth of one apical bud loaded onto a 15% SDS polyacrylamide gel. After electrophoresis the proteins were visualised by silver staining as described in Section 2.6.3.

Lane markings are as follows: (M) purified RUBISCO; (A) extract from plants maintained in darkness for 24 hours; (B) extract from plants maintained in darkness for 48 hours.

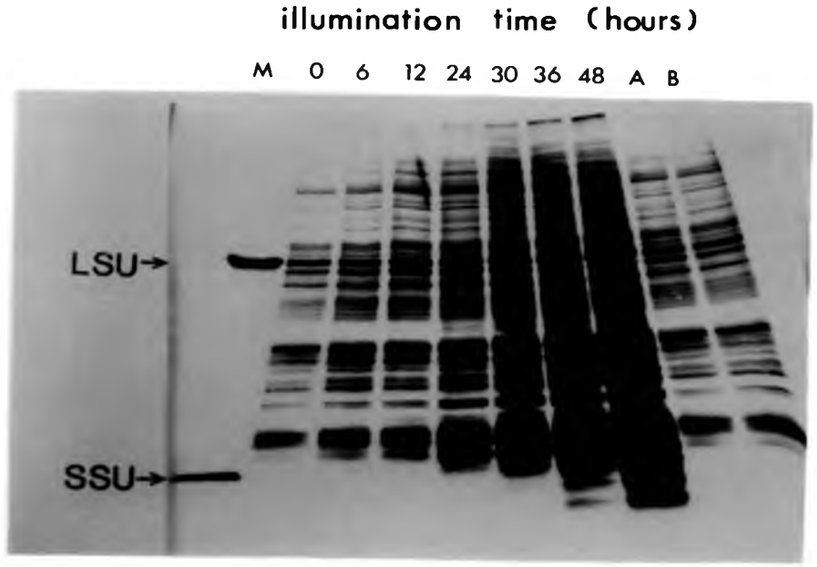


Figure 3.32 - Accumulation of RUBISCO subunits on illumination of pea plants as determined by silver staining. Apical buds were excised from etiolated pea plants, and from etiolated plants which had been subjected to illumination for increasing periods of time. Total soluble protein was extracted and protein equivalent to one twentieth of one apical bud loaded onto a 15% SDS polyacrylamide gel. After electrophoresis the proteins were visualised by silver staining as described in Section 2.6.3.

Lane markings are as follows: (M) purified RUBISCO; (A) extract from plants maintained in darkness for 24 hours; (B) extract from plants maintained in darkness for 48 hours.

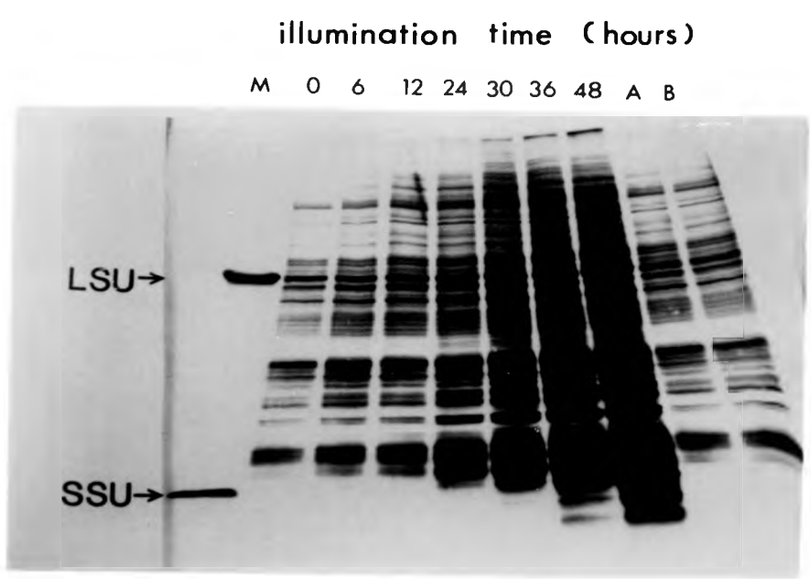


Figure 3.32 - Accumulation of RUBISCO subunits on illumination of pea plants as determined by silver staining. Apical buds were excised from etiolated pea plants, and from etiolated plants which had been subjected to illumination for increasing periods of time. Total soluble protein was extracted and protein equivalent to one twentieth of one apical bud loaded onto a 15% SDS polyacrylamide gel. After electrophoresis the proteins were visualised by silver staining as described in Section 2.6.3.

Lane markings are as follows: (M) purified RUBISCO; (A) extract from plants maintained in darkness for 24 hours; (B) extract from plants maintained in darkness for 48 hours.

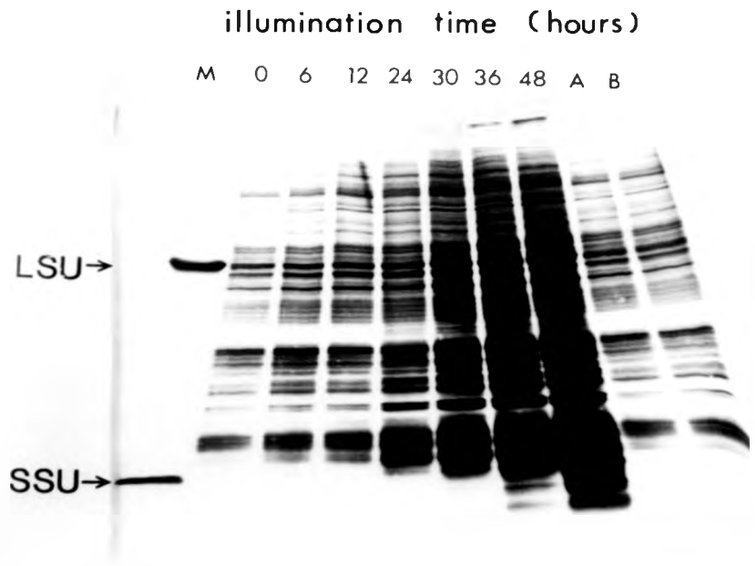


Figure 3.33 - Accumulation of RUBISCO subunits and RUBISCO LSU-binding protein on illumination of etiolated plants as determined by "Western" blotting. Apical buds from plants raised in total darkness for 8 days, and from etiolated plants which had been subjected to increasing periods of illumination, were excised and total soluble protein extracted. Protein equivalent to one twentieth of one apical bud was loaded onto a 15% SDS polyacrylamide gel. After electrophoresis the proteins were transferred onto a nitrocellulose filter and the positions of the RUBISCO subunits and the RUBISCO LSU-binding protein determined by incubating the filter with antiserum raised against the proteins and ^{125}I-labelled protein A (see Section 2.5.3).

Lane symbols are as follows: (M) purified RUBISCO + RUBISCO LSU-binding protein complex; (BP) LSU-binding protein subunit.

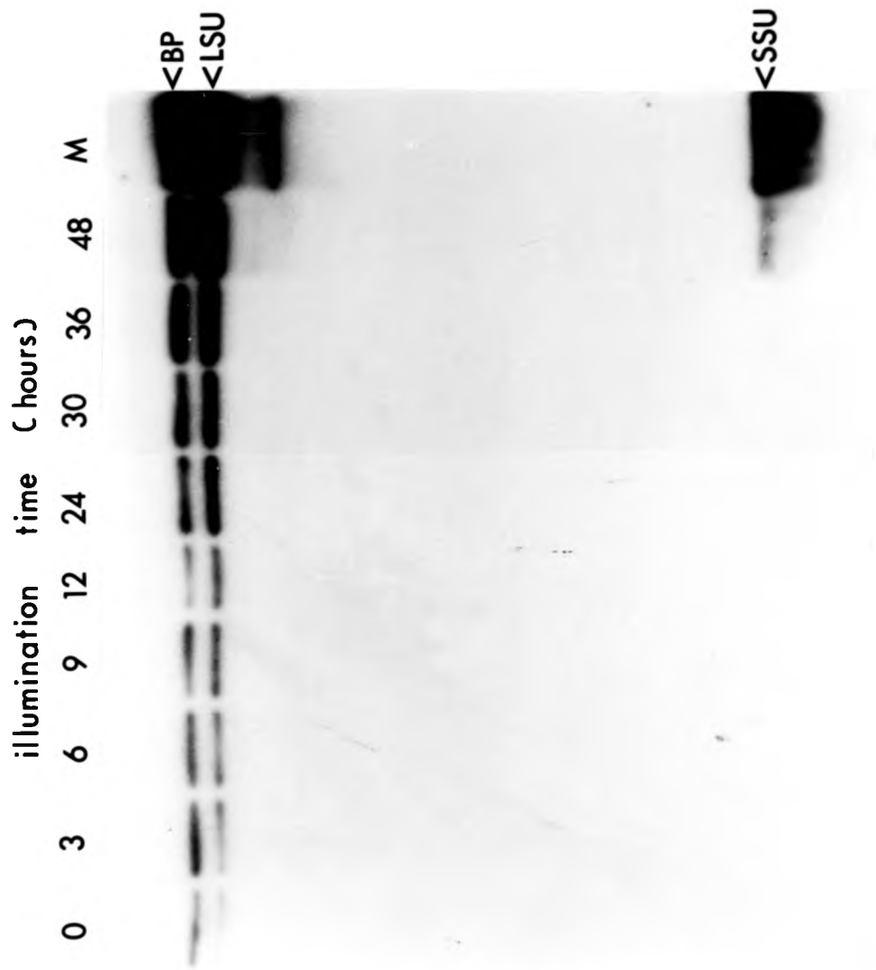
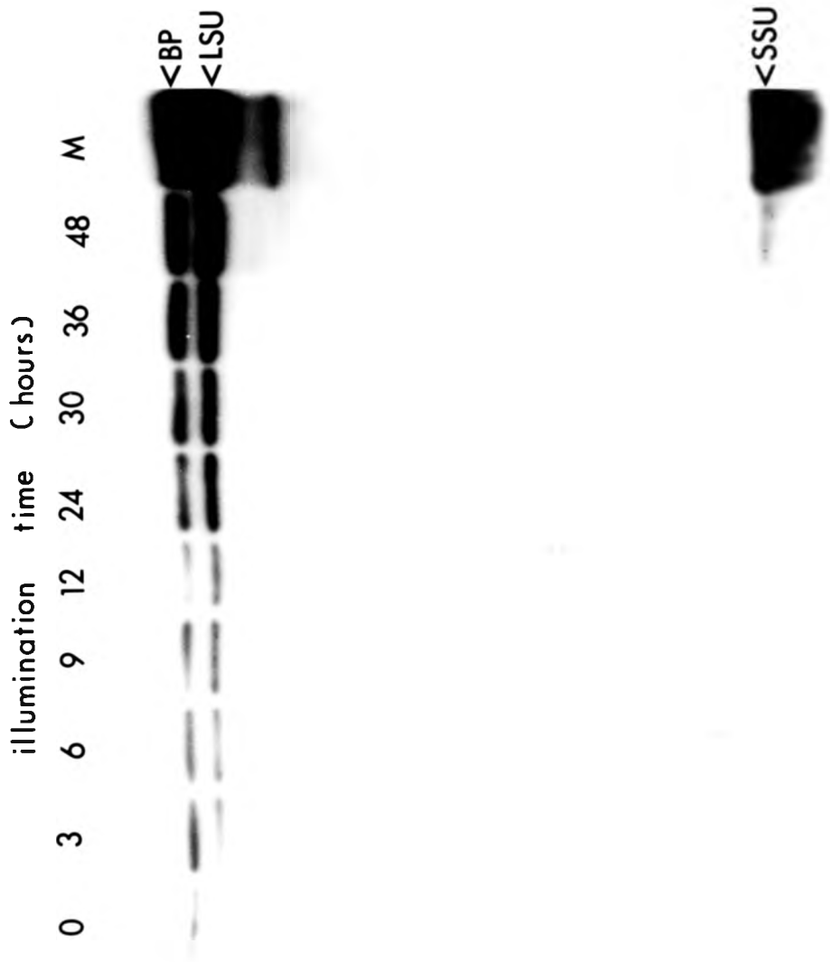


Figure 3.33 - Accumulation of RUBISCO subunits and RUBISCO LSU-binding protein on illumination of etiolated plants as determined by "Western" blotting. Apical buds from plants raised in total darkness for 8 days, and from etiolated plants which had been subjected to increasing periods of illumination, were excised and total soluble protein extracted. Protein equivalent to one twentieth of one apical bud was loaded onto a 15% SDS polyacrylamide gel. After electrophoresis the proteins were transferred onto a nitrocellulose filter and the positions of the RUBISCO subunits and the RUBISCO LSU-binding protein determined by incubating the filter with antiserum raised against the proteins and ^{125}I-labelled protein A (see Section 2.5.3).

Lane symbols are as follows: (M) purified RUBISCO + RUBISCO LSU-binding protein complex; (BP) LSU-binding protein subunit.



the result of the illumination treatment, or was some artefactual finding resulting from the techniques employed, a number of further experiments were performed. A second immunoblot was prepared onto which was loaded protein equivalent to one tenth of one apical bud (Figure 3.34). Both LSU and SSU were found to be present in apical buds from etiolated plants, and both subunits accumulated as the buds were exposed to increasing periods of illumination. Thus the inability to detect SSU in an extract equivalent to one twentieth of one apex appears to be essentially a loading phenomenon. At levels of RUBISCO below 4 µg the serum used preferentially detects LSU (Figures 3.14 and 3.15). In Figure 3.33 amounts of RUBISCO loaded over the shorter illumination periods were equivalent to between 1 µg and 1.5 µg of protein (as determined by rocket immunoelectrophoresis; see Figure 3.31), the very amounts which exhibit differential sensitivities of detection of the subunits of RUBISCO. The silver staining technique was also found to have given misleading results. As shown in Figure 3.35 at levels of RUBISCO below 1.4 µg, LSU is detectable by silver staining while SSU is not.

Bearing in mind the problems involved in immunoblotting, a "Western" blot was performed similar to that shown in Figure 3.34, and this blot was used to provide a quantitative representation of the photoregulation of the RUBISCO subunits and of the LSU-binding protein. The result is shown in Figure 3.36. Amounts of binding protein quadrupled over the period of illumination used, confirming

Figure 3.34 - RUBISCO SSU is present in etiolated pea plants. Apical buds from plants raised in total darkness for 8 days, and from etiolated plants which had been subjected to increasing periods of illumination, were excised and total soluble protein extracted. Protein equivalent to one tenth of one apical bud was loaded onto a 15% SDS polyacrylamide gel. After electrophoresis the proteins were transferred onto a nitrocellulose filter and the positions of the RUBISCO SSU determined by incubating the filter with antiserum raised against the protein and ^{125}I-labelled protein A.

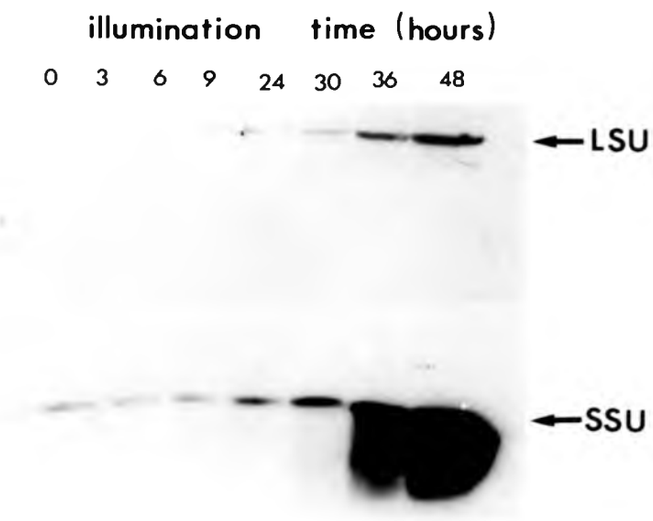


Figure 3.34 - RUBISCO SSU is present in etiolated pea plants. Apical buds from plants raised in total darkness for 8 days, and from etiolated plants which had been subjected to increasing periods of illumination, were excised and total soluble protein extracted. Protein equivalent to one tenth of one apical bud was loaded onto a 15% SDS polyacrylamide gel. After electrophoresis the proteins were transferred onto a nitrocellulose filter and the positions of the RUBISCO SSU determined by incubating the filter with antiserum raised against the protein and ^{125}I-labelled protein A.

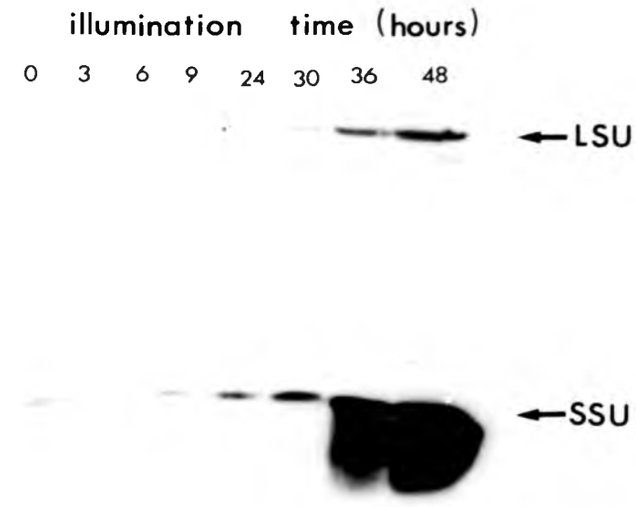


Figure 3.35 - Limits of detection of RUBISCO subunits by silver staining. Purified RUBISCO was subjected to electrophoresis under denaturing conditions and the proteins visualised by silver staining (see Section 2.6.3).

Lane markings are as follows: (a) 0.14 µg; (b) 0.28 µg; (c) 0.42 µg; (d) 0.56 µg; (e) 0.70 µg; (f) 1.40 µg; (g) 2.80 µg; (h) 4.20 µg; (i) 5.60 µg; (j) 7.00 µg; (k) 14.00 µg; (l) 28.00 µg.

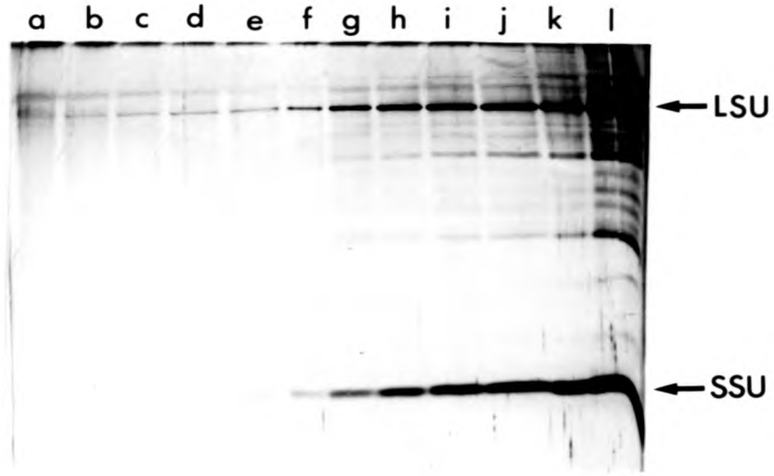


Figure 3.35 - Limits of detection of RUBISCO subunits by silver staining. Purified RUBISCO was subjected to electrophoresis under denaturing conditions and the proteins visualised by silver staining (see Section 2.6.3).

Lane markings are as follows: (a) 0.14 µg; (b) 0.28 µg; (c) 0.42 µg; (d) 0.56 µg; (e) 0.70 µg; (f) 1.40 µg; (g) 2.80 µg; (h) 4.20 µg; (i) 5.60 µg; (j) 7.00 µg; (k) 14.00 µg; (l) 28.00 µg.

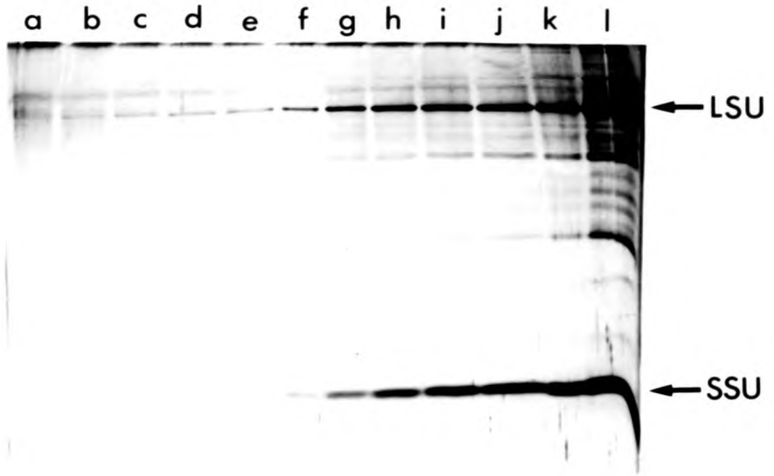
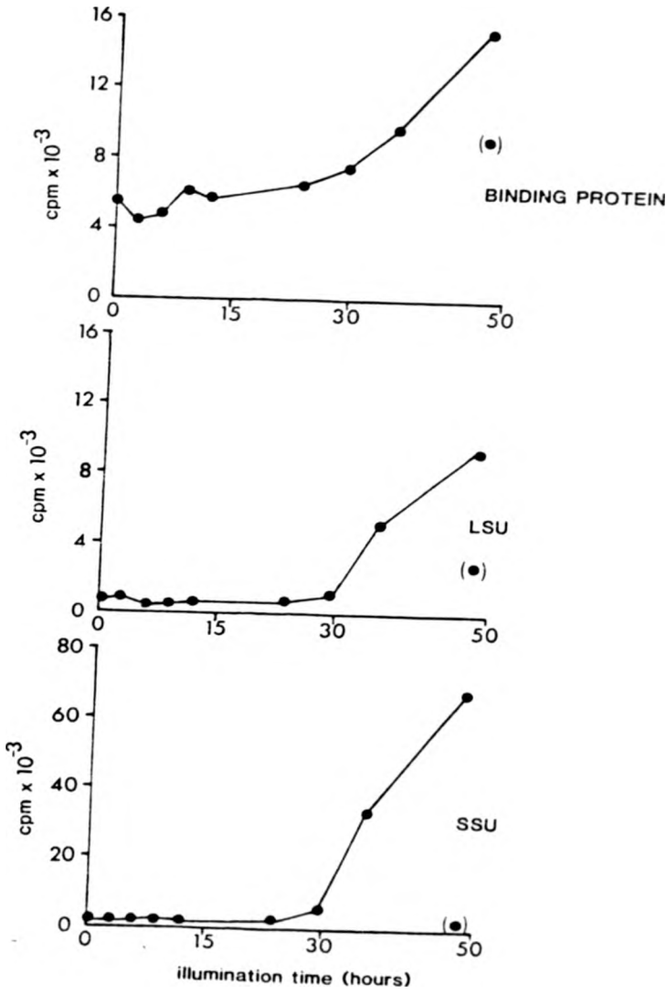


Figure 3.36 - Photoregulation of the RUBISCO subunits and the RUBISCO LSU-binding protein. Apical buds from etiolated plants, and etiolated plants exposed to light for increasing periods of time, were excised and total soluble protein extracted. Protein equivalent to one tenth of one apical bud was subjected to electrophoresis under denaturing conditions and the proteins transferred onto nitrocellulose filter. The positions of the RUBISCO subunits and the RUBISCO LSU-binding protein were determined by incubating the filter with antiserum raised against the proteins and ^{125}I-labelled protein A. The filter was exposed to X-ray film for 16 hours and then bands on the filter corresponding to LSU, SSU and RUBISCO LSU-binding protein were excised and counted for radioactivity. All counts were corrected for background which was obtained from pieces of filter which gave no darkening of film. Counts for LSU, SSU and LSU-binding protein were plotted against illumination time. Symbols in brackets refer to counts for subunits in extracts from plants which had been maintained in total darkness for 48 hours.



the result obtained by rocket immunoelectrophoresis (Figure 3.31). Thus there does not appear to be any particular effect of light on the synthesis of the LSU-binding protein other than that which is responsible for the increased growth of the apex. Both LSU and SSU are present in etiolated tissue but at very low concentrations. The rapid accumulation of RUBISCO observed after 24 hours of illumination (Figure 3.31) appears to be due to an increased synthesis of both LSU and SSU at this time (Figure 3.36). The time-course of synthesis of both LSU and SSU as controlled by light appears to be similar. However, because of the differing responsiveness of the two polypeptides to the antisera used, the data do not allow a determination of the LSU/SSU ratio over the illumination period and therefore the question of coordination in terms of molar ratios of the subunits cannot be addressed. There does, however, appear to be a greater effect of light on SSU synthesis than on the synthesis of LSU from 24 hours of illumination onwards; the amounts of SSU appear to increase by 30-fold while the LSU increase is 15-fold. Over the range of RUBISCO amounts which had been loaded onto the gel, the immunoblot of which is shown in Figure 3.36 (5 µg - 30 µg as determined by rocket immunoelectrophoresis), there was an approximate linear relationship between the amount loaded onto the gel and the counts detected for each subunit on the immunoblot (Figure 3.15). These increases were therefore representative of the subunit accumulation. No correlation between the synthesis of the LSU-binding protein and either of the RUBISCO subunits

was observed.

3.3.3 Activity of RUBISCO in etiolated and light-grown plants

Having established that both LSU and SSU are present in etiolated Pisum apices it was of interest to determine if holoenzyme was formed and, if so, if it was enzymically active. Both LSU and SSU were found to sediment at the position corresponding to RUBISCO in a sucrose density gradient (Figure 3.37). The binding protein in extracts from etiolated plants sediments slightly faster than the RUBISCO holoenzyme as in the extracts from light-grown plants (see Figure 3.24).

The RUBISCO which is present in etiolated Pisum apices has the same specific activity as that present in apices from plants grown under a 12-hour photoperiod (50 and 47 nmoles CO_2 fixed per min per mg RUBISCO respectively). The RUBISCO activities determined were directly proportional to the amount of stromal protein present in the assay (Figure 3.38) and the reaction was not substrate-limited (Figure 3.39), the RuBP concentration employed being 0.5mM. The amount of RUBISCO in each assay was determined by rocket immunoelectrophoresis.

The finding that RUBISCO in dark-grown plants has an identical specific activity to that seen in light-grown plants is in direct contrast to the early work of Smith et al. (1974) who found that RUBISCO protein was present

Figure 3.37 - The presence of RUBISCO holoenzyme in extracts from etiolated plants. Total soluble protein was extracted from 10-day old etiolated plants and this protein was loaded onto a 5%-50% (w/v) sucrose step gradient. After centrifugation (see Section 2.7.4), selected fractions were subjected to electrophoresis on a 15% SDS polyacrylamide gel. After electrophoresis the proteins were transferred onto a nitrocellulose filter, and the positions of the RUBISCO subunits and the RUBISCO LSU-binding protein subunit determined by incubating the filter with antisera to all three subunits and ^{125}I-labelled protein A. The nitrocellulose was then exposed to X-ray film for 16 hours. (A) shows selected fractions of the gradient and (B) shows three of these fractions which have been loaded onto the gel at twice the loading. Markers are as follows: (M) purified RUBISCO and RUBISCO LSU-binding protein complex; (a) RUBISCO LSU-binding protein subunit; (b) LSU; (c) SSU.
The top and bottom of the gradient are marked.

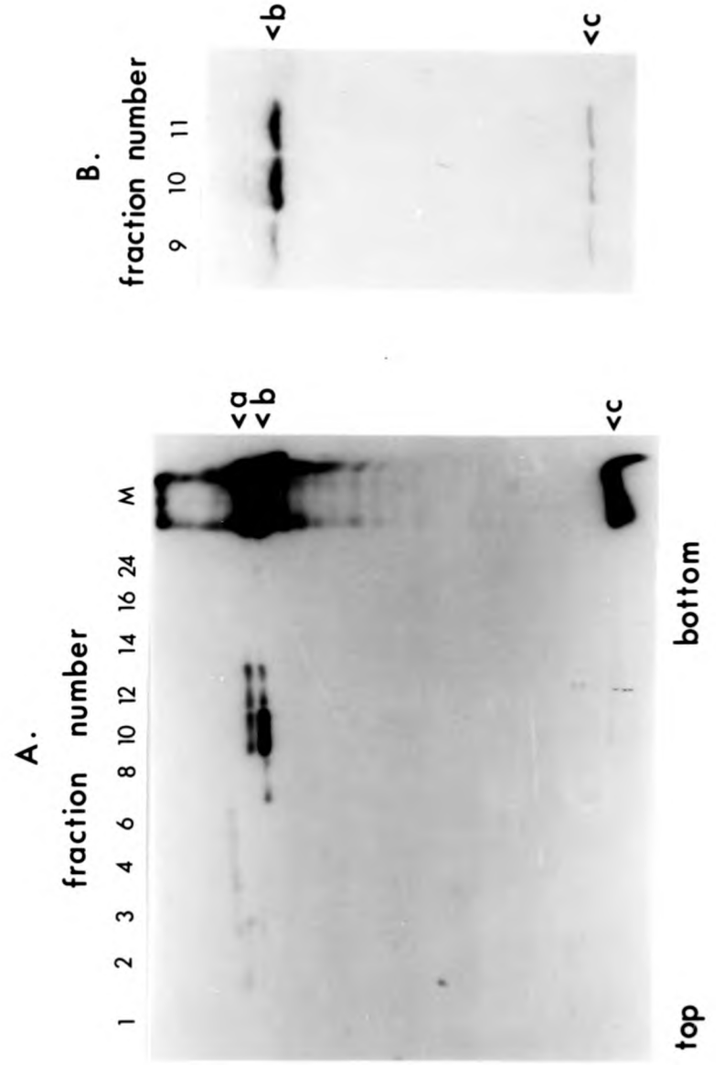


Figure 3.37 - The presence of RUBISCO holoenzyme in extracts from etiolated plants. Total soluble protein was extracted from 10-day old etiolated plants and this protein was loaded onto a 5%-50% (w/v) sucrose step gradient. After centrifugation (see Section 2.7.4), selected fractions were subjected to electrophoresis on a 15% SDS polyacrylamide gel. After electrophoresis the proteins were transferred onto a nitrocellulose filter, and the positions of the RUBISCO subunits and the RUBISCO LSU-binding protein subunit determined by incubating the filter with antisera to all three subunits and ^{125}I-labelled protein A. The nitrocellulose was then exposed to X-ray film for 16 hours. (A) shows selected fractions of the gradient and (B) shows three of these fractions which have been loaded onto the gel at twice the loading. Markers are as follows: (M) purified RUBISCO and RUBISCO LSU-binding protein complex; (a) RUBISCO LSU-binding protein subunit; (b) LSU; (c) SSU.
The top and bottom of the gradient are marked.

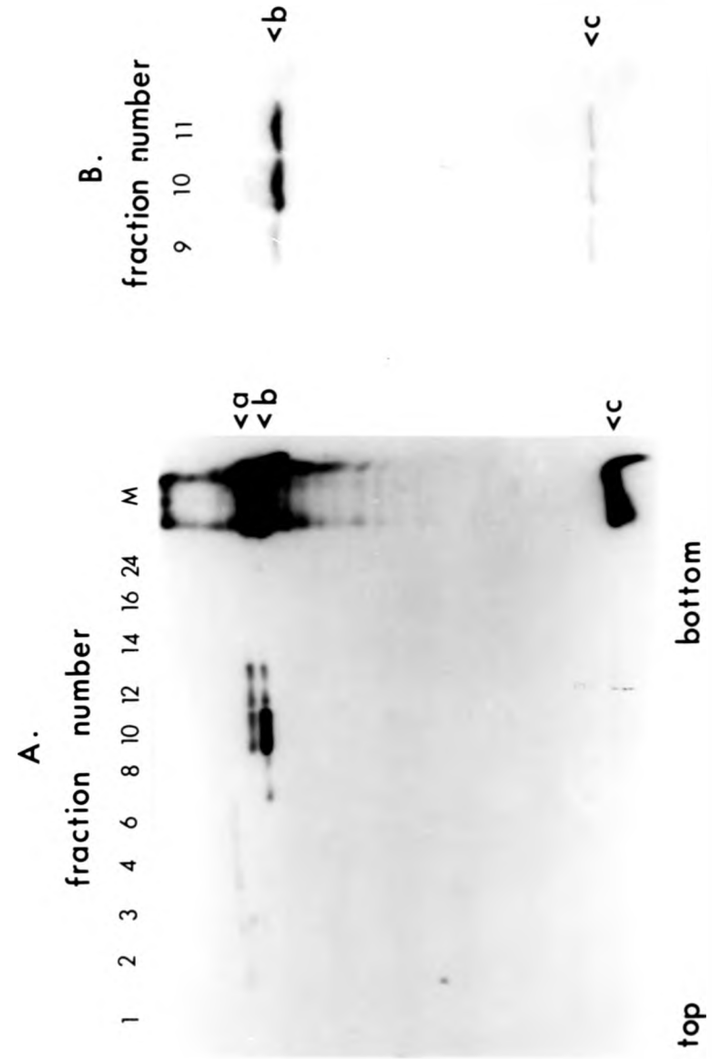


Figure 3.38 - The dependence of RUBISCO activity on the amount of stromal protein present. Stromal protein was extracted from eight-day old pea plants and RUBISCO activity was determined at increasing concentrations of stromal protein as described in Section 2.7.3.

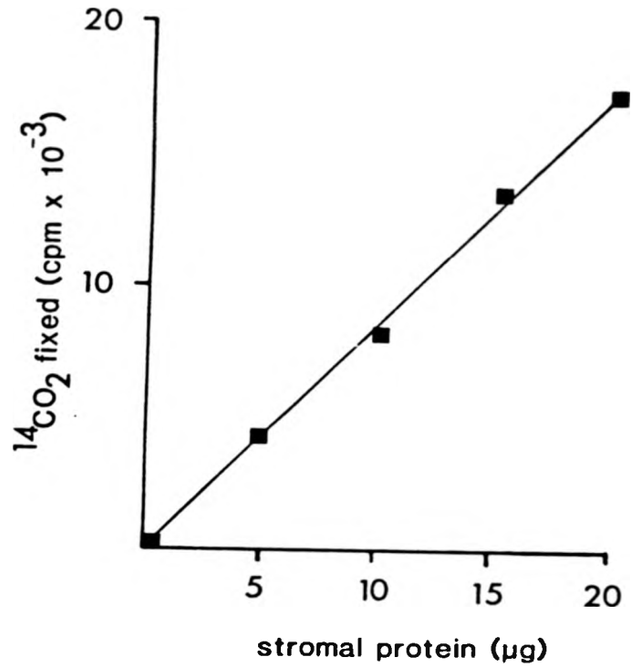
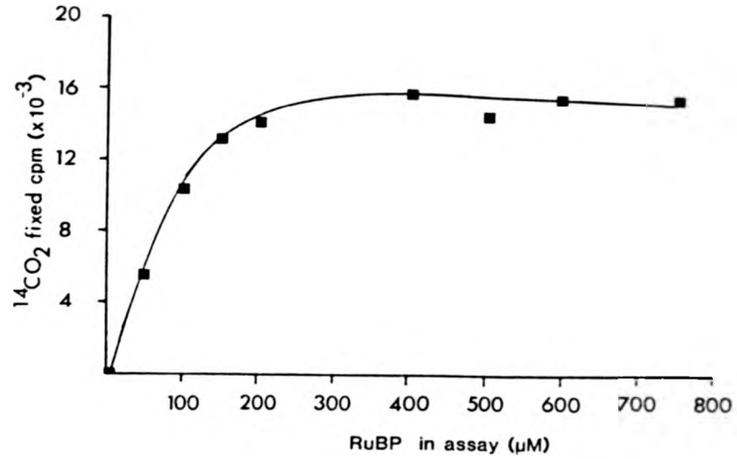


Figure 3.39 - The dependence of RUBISCO activity on the concentration of RuBP present. Stromal protein was extracted from eight-day old pea plants and RUBISCO activity was determined at increasing concentrations of RuBP as described in Section 2.7.3.



in etiolated and greening Hordeum plants before any RUBISCO activity was detectable. These workers proposed a model in which both RUBISCO subunits were present in etiolated plants and etiolated plants exposed to light for a short period, but that these subunits did not become associated to form an active RUBISCO until much later in the illumination period. This is clearly not so in the case of Pisum sativum. The subunits which are synthesised in etiolated Pisum plants appear to be present in an L_8S_8 form, as determined by the sedimentation behaviour, and this form is as enzymically active as the L_8S_8 form of the RUBISCO found in light-grown Pisum plants.

3.3.4 Discussion

The presence of LSU in etiolated Pisum plants is consistent with the findings of Jenkins et al., (1983) and Bennett et al., (1984) who detected LSU polypeptide by immunoblotting, and found that amounts of this protein increased markedly after about 24 hours of illumination of 8-day old dark-grown plants. These workers also measured the changes in LSU mRNA levels on illumination of etiolated plants, and found that LSU transcripts were present in dark-grown plants, and that the concentration of these began to increase during the first 12 hours of illumination. It was therefore suggested that, as the accumulation of the LSU polypeptide lags behind that of the mRNA, the control of LSU synthesis does not only take place at the level of

transcription (Jenkins *et al.*, 1984). Evidence that translational control of some description may be exerted over the synthesis of LSU has been presented by Inamine *et al.* (1985). These workers have shown that on illumination of etiolated *Pisum* plants there is an increase in LSU mRNA which is similar in magnitude to the light-induced increase in chloroplast genome copy number. However, this increase in LSU mRNA amount was not large enough to account for the observed increase in LSU present within the carboxylase holoenzyme, suggesting the involvement of translational control(s).

Bennett *et al.* (1984) have shown that SSU mRNA is detectable in etiolated *Pisum* plants, but that the concentration remains low until the plants have been subjected to 24 hours of illumination. Despite the findings of Jenkins *et al.* (1983), who found, by immunoblotting, that SSU polypeptide is at or below the limits of detection in etiolated plants, it is clear from Figures 3.35 and 3.36 that the low level of SSU mRNA present in etiolated plants is translated *in vivo*. Sasaki *et al.*(1981) have shown that SSU mRNA isolated from etiolated *Pisum* plants can be translated *in vitro* in a wheat germ translation system and that the resulting SSU precursor can be detected on polyacrylamide gels. Thus it would appear that light does not act by "switching on" SSU gene expression, but rather light amplifies that expression which is already occurring at a low level in etiolated plants.

Consistent with the results of Figure 3.36,

Bennett *et al.* (1984) have shown that SSU mRNA concentrations begin to rise after etiolated plants have been exposed to 24 hours of illumination. Control of SSU gene expression appears to be exerted at the transcriptional level (Gallagher & Ellis, 1982; Silverthorne & Tobin, 1984; Berry-Lowe & Meagher, 1985). Transcription of SSU genes in nuclei isolated from light-grown plants is eighteen-fold higher than in nuclei isolated from etiolated plants. The transcripts so formed are stable *in vitro*, suggesting that light acts by increasing the transcription of the gene rather than by decreasing the rate of transcript degradation (Gallagher & Ellis, 1982).

Differences in the mechanisms controlling the accumulation of RUBISCO in different species have been observed. In *Zea mays*, both LSU and SSU are readily detectable in dark-grown plants, (Nelson *et al.*, 1984). On illumination of etiolated plants, SSU is seen to increase in amount by two-fold while LSU increases by a factor of one and a half. A "Western" blot analysis of the accumulation of the subunits of RUBISCO by these workers revealed that LSU was synthesised before the synthesis of SSU began. In the light of the work reported above it is possible that this inability to detect SSU may be an artefact of the method used.

SECTION FOUR - CONCLUSIONS

4.1 PROPERTIES OF THE LSU-BINDING PROTEIN

The work described in this thesis serves to confirm the results of earlier studies on the LSU-binding protein, but also extends the information available concerning the biochemistry of this protein; it is important to gain more knowledge of this protein if its function is to be unequivocally determined.

The interesting observation that the LSU-binding protein is present in two distinct interchangeable forms in chloroplast stromal extracts (see Section 3.2.4) is one which may lead to an understanding of the physiological function of the LSU-binding protein; a number of the salient points regarding this property of the LSU-binding protein are emphasised here. The two forms of the LSU-binding protein observed in stromal extracts, the monomeric form and an oligomeric form, appear to be in equilibrium with each other, and the position of the equilibrium is determined by the concentration of MgATP in the chloroplast stroma. The unassembled LSUs appear to be associated only with the oligomeric form of the binding protein; there is no evidence from the present work that the LSUs remain associated with the binding protein subunits on dissociation by MgATP, and indeed, as the MgATP concentration is increased, it is binding protein monomers which appear to be released from the oligomeric complex (see Figure 3.22). The question as to the fate of the unassembled LSUs on dissociation of the oligomer

has not been resolved. A number of proposals concerning this problem have appeared in the literature and these will be discussed in Section 4.3 below, in a consideration of possible functions of the binding protein.

It seems probable that under natural environmental conditions of illumination much of the binding protein will be in the monomeric form *in vivo*, as the stromal concentration of ATP is usually greater than 1 mM (e.g. Hampp *et al.*, 1982). Upon illumination, and as a consequence of photosynthetic photophosphorylation, the ATP concentration within the chloroplast almost doubles. The stromal concentration of magnesium ions is also thought to increase upon illumination of chloroplasts (Barber, 1976). Efflux of magnesium from the thylakoids is thought to occur to allow maintenance of bulk electrical neutrality of the chloroplast, as light-stimulated proton uptake into the intrathylakoid space proceeds. The dissociation of the binding protein oligomer will therefore be more complete under conditions of illumination, when the requirement for increased ATP and magnesium ion concentrations can be met. Conversely, on transfer of plants to darkness, the equilibrium between the monomeric and oligomeric forms of binding protein will shift towards the oligomeric form as ATP concentration falls, and magnesium ions move back into the thylakoid space.

The finding that the ATP-dependent dissociation of the oligomeric complex is reversible (see Section 3.2.4) raises the question as to how MgATP can effect this. Other

studies have reported that no covalent modification of the binding protein takes place upon dissociation (Hemmingsen & Ellis, 1986). Inability to demonstrate such a modification does not, however, preclude such a possibility; it may have been that, for example, the techniques employed were not sufficiently sensitive in the search for modified subunits of binding protein. The finding that a non-hydrolysable analogue of ATP is ineffective in bringing about dissociation of the oligomer (Bloom et al., 1983) suggests that ATPase activity may be involved. However, it may be that the differing stereochemistry of such an analogue prevents a conformational change in the binding protein subunits of the oligomeric complex and hence the resulting dissociation (Hemmingsen & Ellis, 1986).

Reversible dissociation of an oligomer which is driven by an ATP-dependent conformational change and which also requires the hydrolysis of ATP is exemplified by the process of muscle contraction (reviewed by Offer, 1976). Muscle contraction depends upon the interaction of filaments of both myosin and actin in the so-called "cross-bridges" observed in striated muscle. The myosin molecules possess an ATPase activity which is stimulated in the presence of actin. The actomyosin complex is dissociated on binding of ATP to the myosin filaments. The ATP is subsequently hydrolysed in what is termed the "recovery stroke" of muscle contraction and reassociation of actin with the myosin results in the release of ADP and inorganic phosphate, and the concomitant "working stroke", or contraction, of the muscle. Further ATP

binding allows the cycle to continue. Neither the actin nor the myosin are covalently modified in the series of reactions and magnesium is known to be essential to the process. On reassociation of actin with the myosin, no ATP formation is observed; further ATP is supplied from creatine phosphate.

A most important line for future research with respect to the properties of the LSU-binding protein will be an investigation into the mechanism(s) of dissociation of the LSU-binding protein oligomer. In particular, it is initially important to determine if hydrolysis of ATP occurs upon oligomer dissociation. A solution to this problem may assist in determining whether or not a covalent modification of the binding protein monomers takes place.

4.2 SYNTHESIS OF THE LSU-BINDING PROTEIN

One of the principal aims of the work described in this thesis was to determine if there is any correlation between the accumulation of the LSU-binding protein and either of the subunits of RUBISCO. The results suggest that there is none (see Section 3.3.2), but this finding may prove useful in assigning a function to the binding protein, especially when it is considered in concert with recent findings regarding the photocontrol of accumulation of LSU.

It may be significant that the binding protein is present in etiolated plants and that its accumulation is not strongly photoregulated. The increase in binding protein observed upon illumination is similar in magnitude to that

observed by others for the DNA content of pea apices, and would, therefore, appear to be an effect on nuclear gene dosage, that is the stimulation of cell division by light. In contrast, recent work regarding the photocontrol of accumulation of RUBISCO LSU has suggested two possible levels of control. The first is a light-regulated effect on gene dosage, in which illumination causes an increase in the LSU gene copy number per cell (Sasaki et al., 1984; Inamine et al., 1985), while the second involves some form of, and as yet unidentified, translational control(s). The latter are implicated in LSU accumulation for two reasons. Firstly, there is an observed lag in the increase of LSU protein on illumination of etiolated plants; although the hybridisable mRNA for the LSU increases steadily, the protein concentration remains low for a time (Jenkins et al. 1984). Secondly, the final observed increase in the accumulated LSU in the RUBISCO holoenzyme cannot be accounted for solely by the observed increase in the LSU mRNA (Inamine et al., 1985). The lag observed in LSU accumulation on illumination of etiolated plants is not attributable to a lack of binding protein, as the latter is present in etiolated plants.

The present work shows that on illumination of etiolated plants there is a burst of accumulation of RUBISCO protein after a lag of 24 hours (see Section 3.3.1 and Figure 3.31). This lag in holoenzyme accumulation coincides with a 24 hour lag before an increase in the accumulation of both LSU and SSU is observed. There are a number of references in the literature concerning the possible control of LSU gene

expression by the SSU. In particular, the work of Sasaki et al. (1981) has shown that the increase in RUBISCO in greening pea shoots parallels the increase in SSU mRNA and protein. Similar results have been presented for the same tissue by Jenkins et al. (1984). The work presented in this report neither proves nor disproves the proposal that SSU can control RUBISCO accumulation by switching on LSU gene expression, but it has revealed that care must be exercised in the use of immunoblots to demonstrate that as SSU accumulation commences, that of LSU accelerates (see Section 3.3.2 and Figures 3.33 and 3.34). The work presented above is, however, in agreement with previous findings that the LSU and SSU proteins accumulate approximately in parallel.

As discussed in Section one, coordination of the synthesis of the RUBISCO subunits can be uncoupled under certain laboratory conditions and evidence does suggest that their synthesis is not tightly coordinated (Ellis, 1981). However, the induction of the accumulation of both subunits, although not their relative rates of accumulation, would appear to be stimulated by light (see Figure 3.36). This suggests that, even although the relative synthesis of each can be uncoupled by, for example, inhibitors of protein synthesis by 80s or 70s ribosomes, there is some way in which the accumulation of both LSU and SSU is regulated, so that a large excess of one or the other does not appear.

The important measurement which has yet to be performed with regard to the control of accumulation of LSU

and SSU by light, is to determine accurately the molar ratios of the two subunits during illumination of etiolated tissue, in order to ascertain if there is a molar excess of one subunit over the other. Earlier studies on this topic seem fraught with the problems of sensitivity of detection. Most workers have employed immunoblotting techniques which show an apparent appearance of one subunit before the other (Jenkins *et al.*, 1983; Nelson *et al.*, 1984). It is clear from the work presented in this thesis that it would be unwise to rely on immunoblotting to arrive at such conclusions (see Figure 3.34). If it does in fact transpire that there is an excess of LSU over SSU in the early stages of greening of etiolated plants, then a role for the binding protein in LSU storage prior to its incorporation into holoenzyme would perhaps be worth considering. However, the possibility that the LSU is artefactually conscripted into a non-functional association with the LSU-binding protein in the absence of SSU is also one which must be considered. These points are considered further in the next section.

4.3 ROLES OF THE LSU-BINDING PROTEIN

A number of roles for the LSU-binding protein have been proposed. It was Ellis *et al.* (1980) who first suggested that the LSU-binding protein complex may store LSUs prior to their assembly into the holoenzyme. In this proposed model, the role of the binding protein may be passive or active. That is, in the first case, the binding protein may

function simply to maintain LSU solubility until combination with SSU. An excess of LSU over SSU may occur in the early stages of greening of etiolated plants, and, if this is the case, then a role for the binding protein in LSU solubility maintenance becomes more attractive. The data reported herein show that SSU is present in etiolated plants, and that this SSU is assembled into enzymically active holoenzyme (see Section 3.3.3 and Figure 3.37). However, the techniques employed do not allow an accurate determination of the relative amounts of LSU and SSU (see Section 3.3.2) and so this proposal remains hypothetical.

An active role for the binding protein in RUBISCO assembly has been proposed by Milos & Roy (1984). These workers claim to have shown that on ATP treatment of stromal extracts the LSU-binding protein complex releases its associated newly-synthesised LSUs which subsequently appear in RUBISCO holoenzyme. All attempts to repeat this finding at Warwick have been unsuccessful (R.J. Ellis, pers. comm.). However, the work described in this thesis shows that on dissociation of the oligomeric LSU-binding protein complex by MgATP, most of the detectable binding protein is present as a monomer (see Figures 3.22 and 3.28). This would suggest that any newly-synthesised LSU associated with the oligomeric complex will be released as free LSUs upon such treatment. These released LSUs may then be available for association with free SSUs for assembly into the RUBISCO holoenzyme. It is possible, however, that smaller oligomers composed of fewer binding protein monomers and newly-synthesised LSUs

result upon ATP-dependent dissociation of the oligomeric complex, and that these smaller oligomers are not detectable by the methods of either Coomassie-blue staining (see Figure 3.22) or immunoblotting (see Figure 3.23) used in this work. If, for example, a binding protein-LSU dimer were to result from dissociation of the oligomeric complex then a more active role for the binding protein in LSU incorporation into RUBISCO holoenzyme may be possible.

Within the chloroplast, under environmental light conditions, there will be a continual import of SSU into the stromal compartment and so it is reasonable to assume that there is a continual flow of LSU into the holoenzyme. If SSU supply is, for any reason, limited then the binding protein may sequester LSU until such time as sufficient SSU is available to allow RUBISCO formation. It is clear that such a sequestration by the binding protein would have to be reversible, to ensure that LSU-availability in the stromal compartment matched that of SSU.

Limitation of supply of SSU within the stromal compartment could occur in two ways. Although it seems unlikely that under normal conditions of illumination the SSU concentration would limit holoenzyme formation, if ATP controls SSU import into the chloroplast then when ATP concentration falls in the dark there may be a concomitant drop in SSU pool size in the chloroplast. Consequently, excess LSU would be sequestered by the binding protein into the oligomeric complex form of the binding protein, as the equilibrium between the two forms of the binding protein is

shifted from the monomeric form to the complex form. However, the possibility that the light/dark changes in ATP concentration will be sufficient to alter SSU pool size by a significant value seems remote; even in the dark the ATP concentration inside the chloroplast is in the range 0.32mM to 0.4mM (Hampp et al., 1982).

A second and more likely possibility may arise from the known transcriptional control of SSU genes by light. Gallagher et al. (1985) have shown that if greened Pisum plants are transferred to darkness, the rate of transcription of the SSU genes drops by 50% within 20 minutes. A drop such as this will presumably result in a reduced SSU protein concentration in the stromal compartment if the period of darkness is sufficiently long. On transfer of plants to darkness the ATP concentration within the chloroplast will drop two-fold (Hampp et al., 1982); this drop may favour recruitment of excess LSUs by binding protein monomers and the formation of the oligomeric LSU-binding protein complex. The effect of light/dark transitions on the amounts of SSU monomer and mRNA concentrations will have to be investigated to prove or disprove this idea. The translation of LSU mRNA has a definite requirement for ATP, but not for light (Siddell & Ellis, 1975) and so changes in ATP concentration may influence the synthesis of LSU also. Thus it will be important to determine the concentration of LSU in plants transferred to darkness.

If the LSU-binding protein is indeed found to function in RUBISCO assembly, whether it be passively or

actively, then the question as to the situation in prokaryotes must be broached. Prokaryotes have no chloroplasts and so RUBISCO assembly may be less complex. For example, in the cyanobacterium Anabaena, it has been shown that the genes for LSU and SSU are cotranscribed (Nierzwicki-Bauer et al., 1984); much progress has been made in obtaining fully assembled and active RUBISCO when genes of cyanobacterial LSU and SSU are cloned and expressed in E. coli (Tabita & Small, 1985; Gatenby et al., 1985). The cyanobacterial LSU is not insoluble when isolated from the holoenzyme and can be reassociated with SSU to give active enzyme, unlike the LSU from eukaryotes (Andrews & Ballment, 1983). This solubility of LSU, coupled with the lack of compartmentation and its associated complexity in the prokaryotes, may obviate the need for a LSU-binding protein in such organisms.

In contrast to the successful work in producing active, reassembled RUBISCO from prokaryotes, a problem which has confounded plant biochemists for a number of years has been their inability to produce an active, eukaryotic RUBISCO from the separated subunits. All attempts to produce enzymically active RUBISCO in soluble systems from its constituent subunits have failed, possibly because of the insolubility of LSU in aqueous media. However, these failures may result because some other plastid factor(s) is neccessary, and this may indeed prove to be the LSU-binding protein. Thus a prime objective for future work is the production of cDNA to the binding protein, with the aim of

expressing binding protein together with LSU and SSU in E. coli. The expression of binding protein concomitantly with LSU and SSU in E. coli may permit RUBISCO assembly. Expression of eukaryotic RUBISCO in prokaryotic cells will, of course, be complicated by the fact that all three subunits may be synthesised as precursors. Both SSU and the binding protein are nuclear encoded in eukaryotes and are known to be synthesised as precursors, which are processed to the mature form within the chloroplast (Robinson & Ellis, 1984b; Hemmingsen & Ellis, 1986). The situation regarding the synthesis of LSU as a precursor is less certain, but if so, then another possibility that awaits investigation is that the binding protein functions to process the LSU precursor to the mature form.

The many steps and components potentially involved in eukaryotic RUBISCO assembly ensure the complexity of this process, and if binding protein can be shown in future work to be necessary for this assembly, there will have been a significant advance in our understanding of the synthesis of this essential photosynthetic enzyme.

SECTION FIVE - REFERENCES

Andrews, T.J. & Abel, K.M. (1981) "Kinetics and subunit interactions of ribulose bisphosphate carboxylase-oxygenase from the cyanobacterium Synechococcus sp.". J. Biol. Chem. 256, 8445-8451.

Andrews, T.J. & Ballment, B. (1983) "The function of the small subunits of ribulose bisphosphate carboxylase-oxygenase". J. Biol. Chem. 258, 7514-7518.

Apel, K. (1979) "Phytochrome-induced appearance of mRNA activity for the apoprotein of the light-harvesting chlorophyll a/b protein". Eur. J. Biochem. 97, 183-188.

Apel, K. (1981) "The protochlorophyllide holochrome of barley (Hordeum vulgare L.)". Eur. J. Biochem. 120, 89-93.

Apel, K. & Kloppstech, K. (1978) "The plastid membranes of barley (Hordeum vulgare). Light-induced appearance of mRNA coding for the apoprotein of the light-harvesting chlorophyll a/b protein". Eur. J. Biochem. 85, 581-588.

Arnon, D. I. (1949) "Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vugaris". Pl. Physiol. 24, 1-15.

Badger, M.R., Kaplan, A. & Berry, J.A. (1980) "Internal inorganic carbon pool of Chamydomonas reinhardtii". Plant

Physiol. 66, 407-413.

Barber, J. (1976) In: "Topics in photosynthesis volume 1: The intact chloroplast" (J. Barber, ed.), pp 89-134. Elsevier, New York.

Barraclough, R. & Ellis, R.J. (1979) "The biosynthesis of ribulose bisphosphate carboxylase. Uncoupling of the synthesis of the large and small subunits in isolated soybean leaf cells". Eur. J. Biochem. 94, 165-177.

Barraclough, R. & Ellis, R.J. (1980) "Protein synthesis in chloroplasts. IX. Assembly of newly-synthesized large subunits into ribulose bisphosphate carboxylase in isolated intact pea chloroplasts". Biochim. Biophys. Acta 608, 19-31.

Bassett, C.L., Rinehart, C.A. & Rawson, J.R.Y. (1985) "Immunological determination of phosphoenolpyruvate carboxylase and the large and small subunits of ribulose 1,5-bisphosphate carboxylase in leaves of the C_4 plant pearl millet". Plant Physiol. 77, 828-832.

Bedbrook, J.R. & Bogorad, L. (1976) "Endonuclease recognition sites mapped on *Zea mays* chloroplast DNA". Proc. Natl. Acad. Sci. USA 73, 4309-4313.

Bedbrook, J.R. & Kolodner, R. (1979) "The structure of

chloroplast DNA". Ann. Rev. Plant Physiol. 30, 593-620.

Bedbrook, J.R., Kolodner, R. & Bogorad, L. (1977) "Zea mays chloroplast ribosomal RNA genes are part of a 22,000 base pair inverted repeat". Cell 11, 739-749.

Bedbrook, J.R., Link, G., Coen, D.M., Bogorad, L. & Rich, A. (1978) "Maize plastid gene expressed during photoregulated development". Proc. Natl. Acad. Sci. USA 75, 3060-3064.

Bedbrook, J.R., Smith, S.M. & Ellis, R.J. (1980) "Molecular cloning and sequencing of cDNA encoding the precursor to the small subunit of chloroplast ribulose-1,5-bisphosphate carboxylase". Nature 287, 692-697.

Bennett, J., Jenkins, G.I. & Hartley, M.R. (1984) "Differential regulation of the accumulation of the light-harvesting chlorophyll a/b complex and ribulose bisphosphate carboxylase/oxygenase in greening pea leaves". J. Cell. Biochem. 25, 1-13.

Berry-Lowe, S.L. & Meagher, R.B. (1985) "Transcriptional regulation of a gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase in soybean tissue is linked to the phyochrome response". Mol. Cell. Biol. 5, 1919-1917.

Berry-Lowe, S.L., McKnight, T.D., Shah, D.M. & Meagher, R.B.

(1982) "The nucleotide sequence, expression, and evolution of one member of a multigene family encoding the small subunit of ribulose-1,5-bisphosphate carboxylase in soybean". J. Mol. Appl. Genet. 1, 483-498.

Blair, G.E. & Ellis, R.J. (1973) "Protein synthesis in chloroplasts. I. Light-driven synthesis of the large subunit of fraction I protein by isolated pea chloroplasts". Biochim. Biophys. Acta 319, 223-234.

Blobel, G. & Dobberstein, B. (1975) "Transfer of proteins across membranes I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma". J. Cell Biol. 67, 835-851.

Bloom, M.V., Milos, P. & Roy, H. (1983) "Light-dependent assembly of ribulose-1,5-bisphosphate carboxlase". Proc. Natl. acad. Sci. USA 80, 1013-1017.

Boffey, S.A. & Leech, R.M. (1982) "Chloroplast DNA levels and the control of chloroplast division in light-grown wheat leaves". Plant Physiol. 69, 1387-1391.

Bottomley, W., Spencer, D., & Whitfeld, P.R., (1974) "Protein synthesis in isolated spinach chloroplasts: comparison of light-driven and ATP-driven synthesis". Arch. Biochem. Biophys. 164, 106-117.

Boulter, D., Ellis, R.J., & Yarwood, A. (1972) "Biochemistry of protein synthesis in plants". Biol. Rev. 47, 113-175.

Bowes, G., Ogren, W.L. & Hageman, R.H. (1971) "Phosphoglycollate production catalysed by ribulose diphosphate carboxylase". Biochem. Biophys. Res. Commun. 45,716-722.

Bowien, B., Mayer, F., Spiess, E., Pahler, A., Englisch, U. & Saenger, W. (1980) "On the structure of crystalline ribulosebisphosphate carboxylase from Alcaligenes eutrophus". Eur. J. Biochem. 106, 405-410.

Bradbeer, J.W. (1981) "Development of photosynthetic function during chloroplast biogenesis". In:"The biochemistry of plants volume 8". (M.D. Hatch & N.K. Boardman, eds.), pp 423-472. Academic Press, London.

Bradford, M.M. (1976) "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding". Anal. Biochem. 72, 248-254.

Briggs, W.R. & Iino, M. (1983) "Blue-light absorbing photoreceptors in plants". Phil. Trans. R. Soc. Lond. B303, 347-359.

Broglie, R., Coruzzi, G., Fraley, R.T., Rogers, S.G., Horsch, R.B., Niedermeyer, J.G., Fink, C.L., Flick, J.S, Chua, N.-H. (1984) "Light-regulated expression of a pea ribulose-1,5-bisphosphate carboxylase small subunit gene in transformed plant cells". Science 224, 838-843.

Burnette, W.N. (1981) "'Western blotting': electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A". Anal. Biochem. 112, 195-203.

Burstein, Y. & Schechter, I. (1977) "Amino acid sequence of the NH_2-terminal extra piece segments of the precursors of mouse immunoglobulin λ_1-type and k-type light chains". Proc. Natl. Acad. Sci. USA 74, 716-720.

Christeller, J.T. & Laing, W.A. (1978) "A kinetic study of ribulose bisphosphate carboxylase from the photosynthetic bacterium Rhodospirillum rubrum". Biochem. J. 173, 467-473.

Chua, N.-H. & Schmidt, G. W. (1978) "Post-translational transport into intact chloroplasts of a precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase". Proc. Natl. Acad. Sci. USA 75, 6110-6114.

Colbert, J.T., Hershey, H.P. & Quail, P.H. (1983)

"Autoregulatory control of translatable phytochrome mRNA levels". Proc. Natl. Acad. Sci. USA 80, 2248-2252.

Corruzi,G., Broglie, R., Cashmore, A. & Chua, N.-H. (1983) "Nucleotide sequences of two pea cDNA clones encoding the small subunit of ribulose 1,5-bisphosphate carboxylase and the major chlorophyll a/b-binding thylakoid polypeptide", J. Biol. Chem. 258, 1399-1402.

Coruzzi, G., Broglie, R., Edwards, C. & Chua, N.-H. (1984) "Tissue-specific and light-regulated expression of a pea nuclear gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase". EMBO J. 3, 1671-1679.

Criddle, R.S., Dau, B., Kleinkopf, G.E. & Huffaker, R.C. (1970) "Differential synthesis of ribulosediphosphate carboxylase subunits". Biochem. Biophys. Res. Comm. 41, 621-627.

Crossland, L.D., Rodermel, S.R. & Bogorad, L. (1984) "Single gene for the large subunit of ribulosebisphosphate carboxylase in maize yields two differentially regulated mRNAs". Proc. Natl. Acad. Sci. USA 81, 4060-4064.

Dean, C. & Leech, R.M. (1982a) "Genome expression during normal leaf development.2.Direct correlation between ribulose bisphosphate carboxylase content and nuclear ploidy in a

polyploid series of wheat". Plant Physiol. 70, 1605-1608.

Dean, C. & Leech, R.M. (1982b) "The co-ordinated synthesis of the large and small subunits of ribulose bisphosphate carboxylase during early cellular development within a seven day wheat leaf". FEBS Letts. 140, 113-116.

Dobberstein, B., Blobel, G. & Chua, N.-H. (1977) "In vitro synthesis and processing of a putative precursor for the small subunit of ribulose-1,5-bisphosphate carboxylase of Chlamydomonas reinhardtii". Proc. Natl. Acad. Sci. USA 74, 1082-1085.

Dron, M., Rahire, M. & Rochaix, J.-D. (1982) "Sequence of the chloroplast DNA region of Chlamydomonas reinhardii containing the gene of the large subunit of ribulose bisphosphate carboxylase and parts of its flanking genes". J. Mol. Biol. 162, 775-793.

Dunsmuir, P. & Bedbrook, J. (1983) "Chlorophyll a/b binding proteins and the small subunit of ribulose bisphosphate carboxylase are encoded by multiple genes in petunia". In: "Structure and Function of Plant Genomes". (O. Ciferri & L. Dure, eds.), pp 221-231. Plenum Press, New York.

Dunsmuir, P., Smith, S. & Bedbrook, J. (1983) "A number of different nuclear genes for the small subunit of RuBPCase are transcribed in petunia". Nucleic Acids Res. 11, 4177-4183.

Dyer, T.A. (1984) "The chloroplast genome: its nature and role in development". In: "Chloroplast Biogenesis". (N.R. Baker & J. Barber, eds.), pp 23-70. Elsevier, Amsterdam.

Ellis, R.J. (1969) "Chloroplast ribosomes: stereospecificity of inhibition by chloramphenicol". Science 163, 477-478.

Ellis, R.J. (1970) "Further similarities between chloroplast and bacterial ribosomes". Planta 91, 329-335.

Ellis, R.J. (1975) "Inhibition of chloroplast protein synthesis by lincomycin and 2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide". Phytochemistry 14, 89-93.

Ellis, R.J. (1977) "Protein synthesis by isolated chloroplasts". Biochem. Biophys. Acta. 463, 185-215.

Ellis, R.J. (1979) "The most abundant protein in the world". Trends Biochem. Sci. 4, 241-244.

Ellis, R.J. (1981) "Chloroplast proteins: synthesis, transport and assembly". Ann. Rev. Plant Physiol. 32, 111-137.

Ellis, R.J. (1983) "Chloroplast protein synthesis: principles and problems". In: "Subcellular Biochemistry, vol. 9" (D.B.

Roodyn, ed.), pp 237-261. Plenum Publishing Corporation.

Ellis, R.J. (1984) "The nuclear domination of chloroplast development". Sci. Prog., Oxf. 69, 129-142.

Ellis, R.J. & Hartley, M.R. (1971) "Sites of synthesis of chloroplast proteins". Nature New Biol. 233, 193-196.

Ellis, R.J. & Hartley, M.R. (1982) "Preparation of higher plant chloroplasts active in protein and RNA synthesis". In: "Methods in Chloroplast Molecular Biology" (M. Edelman, R.B. Hallick & N.-H. Chua, eds.), pp 169-188. Elsevier Biomedical.

Ellis, R.J. & Gatenby, A.A. (1984) "Ribulose bisphosphate carboxylase: properties and synthesis". In: "Annual Proceedings of the Phytochemical Society of Europe vol. 23". (P.J. Lea & G.R. Stewart, eds.), pp 41-60. Clarendon press, Oxford.

Ellis, R.J., Smith, S.M. & Barraclough, R. (1980) "Synthesis, transport and assembly of chloroplast proteins". In: "Genome organisation and expression in plants". (C.J. Leaver, ed.), pp 321-335. Plenum publishing corporation.

Ellis, R.J., Gallagher, T.F., Jenkins, G.I. & Lennox, C.R. (1984) "Photoregulation of the biosynthesis of ribulose bisphosphate carboxylase". J. Embryol.exp. Morph. 83, supplement, 163-178.

Fogg, G.E. (1976) "The growth of plants" Penguin, UK.

Feierabend, J. & Mikus, M. (1977) "Occurence of a high temperature sensitivity of chloroplast ribosome formation in several higher plants". Plant Physiol. 59, 863-867.

Feierabend, J. & Wildner, G. (1978) "Formation of the small subunit in the absence of the large subunit of ribulose 1,5-bisphosphate carboxylase in 70s ribosome-deficient rye leaves". Arch. Biochem. Biophys. 186, 283-291.

Firgaira, F.A., Cotton, R.G.H. & Danks, D.M. (1981) "Isolation and characterization of dihydropteridine reductase from human liver". Biochem. J. 197, 31-43.

Fish, L.E. & Jagendorf, A.T. (1982) "High rates of protein synthesis by isolated chloroplasts". Plant Physiol. 70, 1107-1114.

Freyssinet, G., Eichholz, R.L. & Buetow, D.E. (1984) "Kinetics of accumulation of ribulose-1,5-bisphosphate carboxylase during greening in Euglena gracilis". Plant Physiol. 75, 850-857.

Gallagher, T.F. & Ellis, R.J. (1982) "Light-stimulated transcription of genes for two chloroplast polypeptides in isolated pea leaf nuclei". EMBO J. 1, 1493-1498.

Gallagher, T.F., Jenkins, G.I. & Ellis, R.J. (1985) "Rapid modulation of transcription of nuclear genes encoding chloroplast genes by light". FEBS Letts. 186, 241-245.

Gatenby, A.A. (1984) "The properties of the large subunit of maize ribulose bisphosphate carboxylase-oxygenase synthesised in Escherichia coli". Eur. J. Biochem. 144, 361-366.

Gatenby, A.A., Castleton, J.A. & Saul, N.W. (1981) "Expression in E. coli of maize and wheat chloroplast genes for large subunit of ribulose bisphosphate carboxylase". Nature 291, 117-121.

Gatenby, A.A., van der Vies, S.M. & Bradley, D. (1985) "Assembly in E. coli of a functional multi-subunit ribulose bisphosphate carboxylase from a blue-green alga". Nature 314, 617-620.

Gelvin, S., Heizmann, P. & Howell, S.H. (1977) "Identification and cloning of the chloroplast gene coding for the large subunit of ribulose-1,5-bisphosphate carboxylase from Chlamydomonas reinhardi". Proc. Natl. Acad. Sci. USA 74, 3193-3197.

Gershoni, J.M. & Palade, G.E. (1982) "Electophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to a positively charged membrane filter". Anal. Biochem.

124, 396-405.

Gibson, J.L. & Tabita, F.R. (1977a) "Different molecular forms of D-ribulose-1,5-bisphosphate carboxylase from Rhodopseudomonas sphaeroides". J. Biol. Chem. 252, 943-949.

Gibson, J.L. & Tabita, F.R. (1977b) "Isolation and preliminary characterization of two forms of ribulose 1,5-bisphosphate carboxlase from Rhodopseudomonas capsulata". J. Bacteriol. 132, 818-823.

Gibson, J.L. & Tabita, F.R. (1977c) "Characterization of antiserum directed against form II ribulose 1,5-bisphosphate carboxylase from Rhodopseudomonas sphaeroides". J. Bacteriol. 131, 1020-1022.

Gibson, J.L. & Tabita, F.R. (1979) "Activation of ribulose 1,5-bisphosphate carboxylase from Rhodopseudomonas sphaeroides: probable role of the small subunit". J. Bacteriol. 140, 1023-1027.

Gollmer, I. & Apel, K. (1983) "The phytochrome-controlled accumulation of mRNA sequences encoding the light-harvesting chlorophyll a/b protein of barley (Hordeum vulgare L.)". Eur. J. Biochem. 133, 309-313.

Graham, D., Grieve, A.M. & Smillie, R.M. (1968) "Phytochrome

as the primary photoregulator of the synthesis of Calvin cycle enzymes in etiolated pea seedlings". Nature 218, 89-90.

Gray, J.C. & Kekwick, R.G.O. (1974a) "An immunological investigation of the structure and function of ribulose 1,5-bisphosphate carboxylase". Eur. J. Biochem. 44, 481-489.

Gray, J.C. & Kekwick, R.G.O. (1974b) "The synthesis of the small subunit of ribulose 1,5-bisphosphate carboxylase in the French bean Phaseolus vulgaris". Eur. J. Biochem. 44, 491-500.

Grossman, A., Bartlett, S. & Chua, N.-H. (1980) "Energy-dependent uptake of cytoplasmically synthesized polypeptides by chloroplasts". Nature 285, 625-628.

Gutteridge, S., Sigal, I., Thomas, B., Arentzen,R., Cordova, A. & Lorimer, G. (1984) "A site-specific mutation within the active site of ribulose-1,5-bisphosphate carboxylase of Rhodospirillum rubrum". The EMBO Journal 3, 2737-2743.

Hampp, R., Goller, M. & Ziegler, H. (1982) "Adenylate levels, energy charge and phosphorylation potential during dark-light and light-dark transition in chloroplasts, mitochondria, and cytosol of mesophyll protoplasts from Avena sativa L.". Plant Physiol. 69, 448-455.

Hardy, R.W.F., Havelka, U.D. & Quebedeaux, B. (1978) "The opportunity for and significance of alteration of ribulose 1,5-bisphosphate carboxylase activities in crop production". In: "Photosynthetic carbon assimilation" (H. W. Siegelman & G. Hind, eds.), pp 165-178. Plenum Press, New York.

Hartley, M.R., Wheeler, A. & Ellis R.J. (1975) "Protein synthesis in chloroplasts. V. Translation of messenger RNA for the large subunit of fraction 1 protein in a heterologous cell-free system". J. Mol. Biol. 91, 67-77.

Hartman, F.C., Norton, I.L., Stringer, C.D. & Schloss, J.V. (1978) "Attempts to apply affinity labelling techniques to ribulose bisphosphate carboxylase/oxygenase" In: "Photosynthetic Carbon Assimilation" (H.W. Siegelman & G. Hind, eds.), pp 245-269. Plenum Press, New York.

Haselkorn, R., Fernandez-Moran, H., Kieras, F.J. & van Bruggen, E.F.J. (1965) "Electron microscopic and biochemical characterization of fraction I protein". Science 150, 1598-1601.

Hedrick, J.L. & Smith, A.J. (1968) "Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis". Arch. Biochem. Biophys. 126, 155-164.

Hemmingsen, S.M. & Ellis, R.J. (1986) "Purification and properties of ribulosebisphosphate carboxylase large subunit binding protein". Plant Physiol. In Press.

Herrmann, R.G. (1972) "Do chromoplasts contain DNA? II. The isolation and characterization of DNA from chloroplasts, mitochondria, and nuclei of Narcissus". Protoplasma 74, 7-17.

Highfield, P.E. (1978) "Studies on the synthesis of the small subunit of fraction I protein". Ph.D. thesis, University of Warwick, Coventry.

Highfield, P.E. & Ellis, R.J. (1976) "Protein synthesis in chloroplasts VII. Initiation of protein synthesis in isolated intact pea chloroplasts". Biochim. Biophys. Acta 447, 20-27.

Highfield, P.E. & Ellis, R.J. (1978) "Synthesis and transport of the small subunit of chloroplast ribulose bisphosphate carboxylase". Nature 271, 420-424.

Inamine, G., Nash, B., Weissbach, H. & Brot, N. (1985) "Light regulation of the synthesis of the large subunit of ribulose-1,5-bisphosphate carboxylase in peas: evidence for translational control". Proc. Natl. Acad. Sci. USA 82, 5690-5694.

Ishiye, M., Goto, H., Sasaki, Y. & Kamikubo, T. (1981) "The high isoelectric point of the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase and a possible role for the transit peptide". FEBS Letts. 124, 233-236.

Iwanij, V., Chua, N.-H. & Siekevitz, P. (1975) "Synthesis and turnover of ribulose bisphosphate carboxylase and of its subunits during the cell cycle of Chlamydomonas reinhardtii". J. Cell. Biol. 64, 572-585.

Jenkins, G.I., Hartley, M.R. & Bennett, J.B. (1983) "Photoregulation of chloroplast development: transcriptional, translational and post-translational controls ?". Phil. Trans. R. Soc. Lond. B303, 419-431.

Jenkins, G.I., Gallagher, T.F., Hartley, M.R., Bennett, J.B. & Ellis, R.J. (1984) "Photoregulation of gene expression during chloroplast biogenesis". In: "Advances in photosynthesis research vol. IV" (C. Sybesma, ed.) pp 863-872. Junk Press, The Hague.

Jensen, R.G. & Bahr, J.T. (1977) " Ribulose 1,5-bisphosphate carboxylase-oxygenase". Ann. Rev. Plant Physiol. 28, 379-400.

Johal, S. & Chollet, R. (1980) "Ribulose-1,5-bisphosphate carboxylase/oxygenase: enzymic, physicochemical and nutritional properties". What's new in plant physiology 11,

45-48.

Jordan, D.B. & Ogren, W.L. (1981) "Species variation in the specificity of ribulose bisphosphate carboxylase/oxygenase". Nature 291, 513-515.

Kawashima, M. & Wildman, S.G. (1972) "Studies on fraction 1 protein. IV. Mode of inheritance of primary structure in relation to whether chloroplast or nuclear DNA contains the code for a chloroplast protein". Biochim. Biophys. Acta 262, 42-49.

Kirk, J.T.O. & Tilney-Bassett, R.A.E. (1978) "The Plastids". Elsevier, Amsterdam.

Kleinkopf, G.E., Huffaker, R.C. & Matheson, A. (1970) "Light-induced de novo synthesis of ribulose 1,5-diphosphate carboxylase in greening leaves of barley". Plant Physiol. 46, 416-418.

Kobayashi, H., Asami, S. & Akazawa, T. (1980) "Development of enzymes involved in photosynthetic carbon assimilation in greening seedlings of maize (Zea mays)". Plant Physiol. 65, 198-203.

Kreuzaler, F., Ragg, H., Fautz, E., Kuhn, D.N. & Hahlbrock (1983) "UV-induction of chalcone synthase mRNA in cell suspension cultures of Petroselinum hortense". Proc. Natl.

Acad. Sci. USA 80, 2591-2593.

Kung, S.-D. (1976) "Tobacco fraction one protein: a unique genetic marker". Science 191, 429-434.

Kung, S.-D., Zhu, Y.S. & Shen, G.F. (1982) "Nicotiana chloroplast genome. III. Chloroplast DNA evolution". Theor. Appl. Genet. 61, 73-79.

Laemmli, U.K. (1970) "Cleavage of structural proteins during the assembly of the head of bacteriophage T4". Nature 227, 680-685.

Laing, W.A., Ogren, W.L. & Hageman, R.H. (1974) "Regulation of soybean net photosynthetic CO_2 fixation by the interaction of CO_2, O_2, and ribulose 1,5-bisphosphate carboxylase".Plant Physiol. 54, 678-685.

Lamb, C.J. & Lawton, M.A. (1983) "Photocontrol of gene expression" In: "Encyclopedia of plant physiology (NS) vol. 16A: Photomorphogenesis". (W. Shropshire & H. Mohr, eds.), pp 213-257. Springer-Verlag, Berlin.

Lane, M.D. & Miziorko, H.M. (1978) "Mechanism of action of ribulose bisphosphate carboxylase/oxygenase". In: "Photosynthetic carbon assimilation". (H.W. Siegelman & G. Hind, eds.) pp 19-40. Plenum Press, New York.

Langridge, P. (1981) "Synthesis of the large subunit of spinach ribulose bisphosphate carboxylase may involve a precursor polypeptide". FEBS Letts. 123, 85-89.

Laskey, R.A., Honda, B.M., Mills, A.D. & Finch, J.T. (1978) "Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA". Nature 275, 416-420.

Laurell, C.-B. (1966) "Quantitative studies of proteins by electrophoresis in agarose gel containing antibodies". Anal. Biochem. 15, 45-52.

Leech, R.M., Rusby, M.G. & Thomson, W.W. (1973) "Plastid differentiation, acyl lipid, and fatty acid changes in developing green maize leaves". Plant Physiol. 52, 240-245.

Lennox, C.R. & Ellis, R.J. (1985) "The carboxylase large subunit binding protein: photoregulation and reversible dissociation". Biochem. Soc. Trans. In press.

Link, G. (1982) "Phytochrome control of plastid mRNA in mustard (Sinapis alba L.)". Planta 154, 81-86.

Link, G., Cohen, D.M. & Bogorad, L. (1978) "Differential expression of the gene for the large subunit of ribulose bisphosphate carboxylase in maize leaf cell types". Cell 15, 725-731.

Liu, X. & Jagendorf, A.T. (1984) "ATP-dependent proteolysis in pea chloroplasts". FEBS Letts. 166, 248-252.

Lorimer, G.H. (1981) "Ribulosebisphosphate carboxylase: amino acid sequence of a peptide bearing the activator carbon dioxide". Biochemistry 20, 1236-1240.

Lorimer, G.L., Badger, M.R. & Andrews, T.J. (1976) "The activation of ribulose-1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibria, kinetics, a suggested mechanism, and physiological implications". Biochemistry 15, 529-536.

Lorimer, G.H., Badger, M.R., & Andrews, T.J. (1977) "D-ribulose-1,5-bisphosphate carboxylase-oxygenase. Improved methods for the activation and assay of catalytic activities". Anal. Biochem. 78, 66-75.

Lyttleton, J.W. (1962) "Isolation of ribosomes from spinach chloroplasts". Exp. Cell Res. 26, 312-317.

McFadden, B.A. (1980) "A perspective of ribulose bisphosphate carboxylase/oxygenase, the key catalyst in photosynthesis and photorespiration". Acc. Chem. Res. 13, 394-399.

McFadden, B.A. & Purohit, K. (1978) " Chemosynthetic, photosynthetic and cyanobacterial ribulose bisphosphate carboxylase". In "Photosynthetic Carbon Assimilation" (H.W.

Siegelman & G. Hind, eds.), pp 179-207. Plenum Press, New York.

McIntinosh, L., Poulson, C. & Bogorad, L. (1980) "Chloroplast gene sequence for the large subunit of ribulose bisphosphatecarboxylase of maize". Nature 288, 556-560.

Mains, R.E. & Eipper, B.A. (1976) "Biosynthesis of adrenocorticotropic hormone in mouse pituitary tumor cells". J. Biol. Chem. 251, 4115-4120.

Margulies, M.M. (1971) "Concerning the sites of synthesis of proteins of chloroplast ribosomes and of fraction I protein (ribulose-1,5-diphosphate carboxylase)". Biochem. Biophys. Res. Comm. 44, 539-545.

Meeker, R. & Tewari, K.K. (1980) "Transfer ribonucleic acid genes in the chloroplast deoxyribonucleic acid of pea leaves". Biochemistry 19, 5973-5981.

Milos, P. & Roy, H. (1984) "ATP-released large subunits participate in the assembly of RuBP carboxylase". J. Cell. Biochem. 24, 153-162.

Mishkind, M.L., Wessler, S.R. & Schmidt, G.W. (1985) "Functional determinants in transit sequences: import and partial maturation by vascular plant chloroplasts of the ribulose-1,5-bisphosphate carboxylase small subunit of

Chlamydomonas". J. Cell Biol. 100, 226-234.

Miziorko, H.M. & Lorimer, G.H. (1983) "Ribulose-1,5-bisphosphate carboxylase-oxygenase". Ann. Rev. Biochem. 52, 507-535.

Miziorko, H.M., Behnke, C.E. & Houkom, E.C. (1982) "Protein liganding to the activator cation of ribulosebisphosphate carboxylase". Biochemistry 21, 6669-6674.

Mohr, H. (1972) "Lectures on photomorphogenesis". Springer, New York.

Morelli, G., Nagy, F., Fraley, R.T., Rogers, S.G. & Chua, N.-H. (1985) "A short conserved sequence is involved in the light-inducibility of a gene encoding ribulose 1,5-bisphosphate carboxylase small subunit of pea". Nature 315, 200-204.

Muller, E.D., Chory, J. & Kaplan, S. (1985) "Cloning and characterization of the gene product of the form II ribulose-1,5-bisphosphate carboxylase gene of *Rhodopseudomonas sphaeroides*". J. Bacteriol. 161, 469-472.

Nelson, T., Harpster, M.H., Mayfield, S.P. & Taylor, W.C. (1984) "Light-regulated gene expression during maize leaf development". J. Cell Biol. 98, 558-564.

Nierzwicki-Bauer, S.A., Curtis, S.E. & Haselkorn, R. (1984) "Cotranscription of genes encoding small and large subunits of ribulose-1,5-bisphosphate carboxylase in the cyanobacterium Anabaena 7120". Proc. Natl. Acad. Sci. USA 81, 5961-5965.

Nivison, H.T. & Stocking, C.R. (1983) "Ribulose bisphosphate carboxylase synthesis in barley leaves. A developmental approach to the question of coordinated subunit synthesis". Plant Physiol. 73, 906-911.

Nobel, P.S. & Wang, C.-T. (1970) "Amino acid permeability of pea chlorplasts as measured by osmotically determined reflection coefficients". Biochim. Biophys. Acta 211, 79-87.

Offer, G.W. (1976) "The molecular basis of muscle contraction". In: "Companion to biochemistry". (A.T. Bull, J.R. Lagnado, J.O. Thomas & K.F. Tipton, eds.), pp 623-671. Longman, London.

Oishi, K.K. & Tewari, K.K. (1983) "Characterisation of the gene and mRNA of the large subunit of ribulose-1,5-bisphosphate carboxylase in pea plants". Mol. Cell. Biol. 3, 587-595.

Oudin, J. (1980) "Immunochemical analysis by antigen-antibody

precipitation". Methods Enzymol. 80 , 166-198.

Plumley, F.G. & Schmidt, G.W. (1983) "Rocket and crossed immunoelectrophoresis of proteins solubilized with sodium dodecyl sulfate". Anal. Biochem. 134, 86-95

Ris, H. & Plaut, W. (1962) "Ultrastructure of DNA-containing areas in the chloroplast of Chlamydomonas". J. Cell Biol. 13, 383-391.

Robinson, C. & Ellis, R.J. (1984a) "Transport of proteins into chloroplasts. Partial purification of a chloroplast protease involved in the processing of imported precursor polypeptides". Eur. J. Biochem. 142, 337-342.

Robinson, C. & Ellis, R.J. (1984b) "Transport of proteins into chloroplasts. The precursor of small subunit of ribulose ribulose bisphosphate carboxylase is processed to the mature size in two steps. Eur. J. Biochem. 142, 343-346.

Roy, H, Patterson, R. & Jagendorf, A.T. (1976) "Identification of the small subunit of ribulose 1,5-bisphosphate carboxylase as a product of wheat leaf cytoplasmic ribosomes". Arch. Biochem. Biophys. 172, 64-73.

Roy, H., Valeri, A., Pope, D.H., Rueckert, L. & Costa, K.A. (1978a) "Small subunit contacts in ribulose-1,5-bisphosphate carboxylase". Biochemistry 17, 665-668.

Roy, H., Costa, K.A. & Adari, H. (1978b) "Free subunits of ribulose-1,5-bisphosphate carboxylase in pea leaves". Plant Sci. Letts. 11, 159-168.

Roy, H., Adari, H. & Costa, K.A. (1979) "Characterization of free subunits of ribulose-1,5-bisphosphate carboxylase". Plant Sci. Letts. 16 305-318.

Roy, H., Bloom, M., Milos, P. & Munroe, M. (1982) "Studies on the assembly of large subunits of ribulose bisphosphate carboxlase in isolated pea chloroplasts". J. Cell Biol. 94, 20-27.

Sakano, K., Kung, S.-D. & Wildman, S.G. (1974) "Identification of several chloroplast DNA genes which code for the large subunit of Nicotiana fraction I proteins". Mol. Gen. Genet. 130, 91-97.

Sani, A., De Koning, W., Dijkhuizen, L. & Dow, C.S. (1983) "Studies on the ribulose-1,5-bisphosphate carboxylase". Proc. 4th Int. Symp. Microbial Growth On C_1 Compounds, Minneapolis, USA, 312.

Sasaki, Y., Ishiye, M., Sakihama, T. & Kamikubo, T. (1981) "Light-induced increase of mRNA activity coding for the small subunit of ribulose-1,5-bisphosphate carboxylase". J. Biol. Chem. 256, 2315-2320.

Sasaki, Y., Sakihama, T., Kamikubo, T. & Shinozaki, K. (1983) "Phytochrome-mediated regulation of two mRNAs, encoded by nuclei and chloroplasts of ribulose-1,5-bisphosphate carboxylase/oxygenase". Eur. J. Biochem. 133, 617-620.

Sasaki, Y., Tomoda, Y. & Kamikubo, T. (1984) "Light regulates the gene expression of ribulosebisphosphate carboxylase at the levels of transcription and gene dosage in greening pea leaves". FEBS Letts. 173, 31-35.

Schmidt, G.W. & Mishkind, M.L. (1983) "Rapid degradation of unassembled ribulose 1,5-bisphosphate carboxylase small subunits in chloroplasts". Proc. Natl. Acad. Sci. USA 80, 2632-2636.

Schmidt, G.W., Devillers-Thiery, A., Desruisseaux, H., Blobel, G. & Chua, N.-H. (1979) "NH_2-terminal amino-acid sequences of precursor and mature forms of the ribulose-1,5-bisphosphate carboxylase small subunit from Chlamydomonas reinhardtii". J. Cell Biol. 83, 615-622.

Schreier, P.H., Seftor, E.A., Schell, J. & Bohnert, H.J. (1985) "The use of nuclear-encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant chloroplasts". EMBO J. 4, 25-32.

Servaites, J.C. & Ogren W.L. (1978) "Oxygen inhibition of

photosynthesis and stimulation of photorespiration in soybean leaf cells". Plant Physiol. 61, 62-67.

Shen, G.F., Chen, K., Wu, M. & Kung, S.-D. (1982) "Nicotiana chloroplast genome. IV. N. accuminata has larger inverted repeats and genome size". Mol. Gen. Genet. 187, 12-18.

Shinozaki, K., Sasaki, Y., Sakihama, T. & Kamikubo, T. (1982) "Coordinate light-induction of two mRNAs, encoded in nuclei and chloroplasts, of ribulose 1,5-bisphosphate carboxylase/oxygenase". FEBS Letts. 144, 73-76.

Shinozaki, K., Yamada, C., Takahata, N. & Sugiura, M. (1983) "Molecular cloning and sequence analysis of the cyanobacterial gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase". Proc. Natl. Acad. Sci. USA 80, 4050-4054.

Shively, J.M., Davidson, E. & Marrs, B.L. (1984) "Depression of the synthesis of the intermediate and large forms of ribulose-1,5-bisphosphate carboxylase/oxygenase in Rhodopseudomonas capsulata". Arch. Microbiol. 138, 233-236.

Siddell, S.G. & Ellis, R.J. (1975) "Protein synthesis in chloroplasts. Characteristics and products of protein synthesis in vitro in etioplasts and developing chloroplasts from pea leaves". Biochem. J. 146 675-685.

Silverthorne, J. & Tobin, E.M. (1984) "Demonstration of transcriptional regulation of specific genes by phytochrome action". Proc. Natl. Acad. Sci. USA 81, 1112-1116.

Smith, H. (1975) "Phytochrome and photomorphogenesis". McGraw-Hill, London.

Smith, M.A., Criddle, R.S., Peterson, L. & Hufaker, R.C. (1974) "Synthesis and assembly of ribulosebisphosphate carboxylase enzyme during greening of barley plants". Arch. Biochem. Biophys. 165, 494-504.

Smith, S.M. & Ellis, R.J. (1979) "Processing of small subunit precursor of ribulose bisphosphate carboxylase and its assembly into whole enzyme are stromal events". Nature 278, 662-664.

Smith, S.M. & Ellis, R.J. (1981) "Light-stimulated accumulation of transcripts of nuclear and chloroplast genes for ribulosebisphosphate carboxylase". J. Mol. Appl. Genet. 1, 127-137.

Somerville, C.R. & Somerville, S.C. (1984) "Cloning and expression of the Rhodospirillum rubrum ribulosebisphosphate carboxylase gene in E. coli". Mol. Gen. Genet. 193, 214-219.

Spreitzer, R.J. & Mets, L.J. (1980) "Non-mendelian mutation affecting ribulose-1,5-bisphosphate carboxylase structure and activity". Nature 285, 114-115.

Spreitzer, R.J., Jordan, D.B. & Ogren, W.L. (1982) "Biochemical and genetic analysis of an RuBP carboxylase/oxygenase-deficient mutant and revertants of Chlamydomonas reinhardii". FEBS Lett. 148, 117-121.

Stanier, R.Y., Adelberg, E.A. & Ingraham, J.L. (1978) "General microbiology", 4th edition. Macmillam Press, London.

Steikema, W.J., Wimpee, C.F., Silverthorne, J. & Tobin, E.M. (1983) "Phytochrome control of the expression of two nuclear genes encoding chloroplast proteins in Lemna gibba L. G-3". Plant Physiol. 72, 717-724.

Stiegler, G.L., Matthews, H.M., Bingham, S.E. & Hallick, R.B. (1982) "The gene for the large subunit of ribulose-1,5-bisphosphate carboxylase in Euglena gracilis chloroplast DNA: location, polarity, cloning and evidence for an intervening sequence". Nucleic Acids Res. 10, 3427-3444.

Tabita, F.R. & McFadden, B.A. (1974) "D-ribulose 1,5-diphosphate carboxylase from Rhodospirillum rubrum. II. Quaternary structure, composition, catalytic, and immunological properties". J. Biol. Chem. 249, 3459-3464.

Tassopulu, D. & Kung, S.-D. (1984) "Nicotiana chloroplast genome. 6. Deletion and hot spot - a proposed origin of the inverted repeats". Theor. Appl. Genet. 67, 185-193.

Thomas, B., Penn, S.E., Butcher, G.W. & Galfre, G. (1984) "Discrimination between the red- and far-red-absorbing forms of phytochrome from Avena sativa L.". Planta 160, 382-384.

Thompson, W.F., Everett, M., Polans, N.O., Jorgensen, R.A. (1983) "Phytochrome control of RNA levels in developing pea and mung-bean leaves". Planta 158, 487-500.

Tobin, E.M. (1978) "Light regulation of specific mRNA species in Lemna gibba L. G-3". Proc. Natl. Acad. Sci. USA 75, 4749-4753.

Tobin, E.M. (1981a) "White light effects on the mRNA for the light-harvesting chlorophyll a/b-protein in Lemna gibba L. G-3". Plant Physiol. 67, 1078-1083.

Tobin, E.M. (1981b) "Phytochrome-mediated regulation of messenger RNAs for the small subunit of ribulose 1,5-bisphosphate carboxylase and the light-harvesting chlorophyll a/b-protein in Lemna gibba". Plant Mol. Biol. 1, 35-51.

Tobin, E.M. & Silverthorne, J. (1985) "Light regulation of

gene expression in higher plants". Ann. Rev. Plant Physiol. 36, 569-593.

Tobin, E. M. & Suttie, J.L. (1980) "Light effects on the synthesis of ribulose-1,5-bisphosphate carboxylase in Lemna gibba L. G-3". Plant Physiol. 65, 641-647.

Torres-Ruiz, J.A. & McFadden, B.A. (1985) "Isolation of L_e and L_eS_e forms of ribulose bisphosphate carboxylase/oxygenase from Chromatium vinosum". Arch. Microbiol. 142, 55-60.

Van den Broeck, G., Timko, M.P., Kausch, A.P., Cashmore, A.R., Van Montagu, M. & Herrera-Estrella, L. (1985) "Targeting of a foreign protein to chloroplasts by fusion to the transit peptide from the small subunit of ribulose 1,5-bisphosphate carboxylase". Nature 313, 358-363.

Vaessen, R.T.M.J., Kreike, J. & Groot, G.S.P. (1981) "Protein transfer to nitrocellulose filters. A simple method for quantitation of single proteins in complex mixtures". FEBS Letts. 124, 193-196.

Vierling, E. & Key, J.L. (1985) "Ribulose 1,5-bisphosphate carboxylase synthesis during heat shock". Plant Physiol. 78, 155-162.

Voordouw, G., van der Vies, S.M. & Bouwmeister, P.J. (1984) "Dissociation of ribulose-1,5-bisphosphate

carboxylase/oxygenase from spinach by urea". Eur. J. Biochem. 141, 313-318.

Weber, K. & Osborn, M., (1969) "The reliability of molecular weight determined by dodecyl sulfate polyacrylamide gel electrophoresis". J. Biol. Chem. 244, 4406-4412.

Weissbach, A., Horecker, B.L. & Hurwitz, J. (1956) "The enzymatic formation of phosphoglyceric acid from ribulose diphosphate and carbon dioxide". J. Biol. Chem. 218, 759-810.

Wildman, S.G. & Bonner, J. (1947) "The proteins of green leaves. I. Isolation, enzymatic properties and auxin content of spinach cytoplasmic proteins". Arch. Biochem. 14, 381-413.

Wray, W., Boulikas, T., Wray, V.P. & Hancock, R. (1981) "Silver staining of proteins in polyacrylamide gels". Anal. Biochem. 118, 197-203.

Zhu, Y.S., Duvall, E.J., Lovett, P.S. & Kung, S.-D. (1982) "*Nicotiana* chloroplast genome. V. Construction, mapping and expression of clone library of *N. otophora* chloroplast DNA". Mol. Gen. Genet. 187, 61-66.

Zhu, Y.S., Lovett, P.S., Williams, D.M. & Kung, S.-D. (1984) "*Nicotiana* chloroplast genome. 7. Expression in *E. coli*

and B. subtilis of tobacco and Chlamydomonas chloroplast DNA sequences coding for the large subunit of RuBP carboxylase". Theor. Appl. Genet. 67, 333-336.

Zurawski, G., Perrot, B., Bottomley, W. & Whitfeld, P.R. (1981) "The structure of the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase from spinach chloroplast DNA". Nucleic Acids Res. 9, 3251-3270.

APPENDIX ONE - PUBLICATIONS

J. Embryol. exp. Morph. 83, Supplement, 163–178 (1984)
Printed in Great Britain © *The Company of Biologists Limited 1984*

Photoregulation of the biosynthesis of ribulose bisphosphate carboxylase

By R. JOHN ELLIS, THOMAS F. GALLAGHER, GARETH I. JENKINS AND C. RUTH LENNOX

Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, U.K.

TABLE OF CONTENTS

Summary
Introduction
The synthesis of Rubisco
Expression of Rubisco genes during greening
The photoregulation of transcription
References

SUMMARY

Chloroplast development in higher plants is light dependent, and is accompanied by the synthesis of chlorophyll and the accumulation of many chloroplast polypeptides. There is a 100-fold greater content of the photosynthetic enzyme, ribulose-1,5-bisphosphate carboxylase-oxygenase, in light-grown seedlings of *Pisum sativum* than in dark-grown seedlings. Following the illumination of dark-grown seedlings, there is a parallel increase in the content of both the mRNA and the polypeptide of the small subunit of the carboxylase; this subunit is a product of the nuclear genome. The increases in the mRNA and the polypeptide of the large subunit, which is a product of the chloroplast genome, show less synchronicity. Studies with isolated leaf nuclei show that the increase in small subunit mRNA is mediated primarily at the level of transcription. Three distinct effects of light on transcription of small subunit genes have been found; a rapid (~1 h) burst, followed by a decline, when etiolated plants are first exposed to light; a slow (~36 h) development of the competence to transcribe rapidly after the initial burst; rapid (~20 min) switches in both directions when fully greened plants are exposed to light–dark transitions.

INTRODUCTION

One of the principal characteristics of green plants which set them apart from animals is that they are photoautotrophic; they use the energy of light to convert carbon dioxide into carbohydrate through the reactions of photosynthesis. This process, which is the mainstay of life on this planet, takes place in chloroplasts (Kirk & Tilney-Bassett, 1978). These organelles are bounded by a double

envelope, and contain thylakoid membranes which house the photosynthetic pigments and electron transport components, and a hydrophilic matrix termed the stroma, which contains the enzymes involved in carbon dioxide fixation. Ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) is of pivotal importance since it catalyses the first step in the assimilation of carbon dioxide (Lorimer, 1981); it is also the most abundant protein in the leaves of higher plants (Ellis, 1979).

The programming of development in plants is very similar, at a fundamental level, to that in animals. In both cases the growth and differentiation of the whole organism, and of its constituent tissues and cells, involves differential gene expression. Our major interest is to understand the molecular basis of this process, and we have concentrated much of our effort on Rubisco because of its evident importance and abundance. This protein contains two types of subunit; a large subunit which is encoded in chloroplast DNA and synthesized within the organelle, and a small subunit which is encoded in nuclear DNA and synthesized on cytoplasmic ribosomes in precursor form prior to being transported into the chloroplast (Ellis, 1981, 1983). Thus an additional attraction of this protein is that it provides an opportunity to investigate the activities of two different genetic systems occurring within the same cell.

One way in which the programming of development in plants differs from that in animals is that, in general, it is influenced to a greater extent by environmental factors, one of the most important being light (Mohr & Shropshire, 1983; Jenkins, 1984). Leaf development for example, is arrested at an early stage in dark-grown plants and is completed only following exposure to light. The formation of mature, photosynthetically-active chloroplasts is also light dependent (Bradbeer, 1981). This requirement for light is hardly surprising since it would be wasteful for the plant, in view of the major investment of seed resources required, to synthesize its photosynthetic machinery under conditions in which photosynthesis could not occur. During normal leaf development in higher plants, that is under either continuous illumination or in a day–night cycle, chloroplasts develop from rudimentary organelles termed proplastids. If, however, plants are grown in darkness the proplastids develop into etioplasts, organelles which are smaller than chloroplasts, and which lack chlorophyll and the well-differentiated thylakoid membrane system typical of chloroplasts. Following illumination of dark-grown plants, etioplasts develop into chloroplasts, and it is this transformation (termed 'greening') which, for experimental convenience, is most frequently used in studies of chloroplast biogenesis.

The light-induced conversion of etioplasts into chloroplasts is accompanied by dramatic changes in molecular composition. Although some components decrease in abundance following illumination, the great majority, including the various pigments, quinones and proteins of the photosynthetic apparatus, accumulate (Bradbeer, 1981). Among the proteins which increase markedly in amount is Rubisco. It should be noted however, that light is not required for the

accumulation of this protein in all cases; Rubisco can be easily detected in dark-grown plants of a number of species, especially cereals. The effect of light is therefore stimulatory rather than absolute, the extent of the stimulation varying between species (Ellis, 1983).

The effect of light on the abundance of Rubisco must involve some system for the detection and transduction of the light signal which results in a change in gene expression. The aim of our research is to address two broad sets of questions. First, at what levels is the expression of genes encoding the large and small subunit polypeptides of Rubisco controlled, what molecular mechanisms are involved, and to what extent are the expressions of the genes for the two subunits co-ordinated? Second, which photoreceptor pigments mediate the effect of light, and what mechanisms link photoreception to events concerned with gene expression?

The availability of cloned DNA hybridization probes for both the large subunit (Coen, Bedbrook, Bogorad & Rich, 1977) and the small subunit (Bedbrook, Smith & Ellis, 1980) has resulted in a number of detailed studies of the effect of light on the expression of the corresponding genes in species such as *Pisum sativum* (Smith & Ellis, 1981; Gallagher & Ellis, 1982; Jenkins, Hartley & Bennett, 1983; Gallagher, Jenkins, Smith & Ellis, 1984; Jenkins *et al.* 1984; Bennett, Jenkins & Hartley, 1984; Sasaki, Sakihama, Kamikubo & Shinozaki, 1983; Thompson *et al.* 1983) and *Lemna gibba* (Tobin, 1981; Stiekema, Wimpee, Silverthorne & Tobin, 1983). In this paper we summarize the conclusions of these studies, and present the results of our latest experiments on the photoregulation of genes involved in the synthesis of Rubisco in *Pisum sativum*.

THE SYNTHESIS OF RUBISCO

Fig. 1 summarizes some of the events believed to be involved in the synthesis of Rubisco in higher plants. In all eukaryotes, and most, but not all, prokaryotes, each molecule of Rubisco is an oligomer of sixteen subunits of two basic types, termed large and small (Miziorko & Lorimer, 1983). The large subunits (relative molecular mass (M_r) about 52 000) carry the active sites for both carboxylase and oxygenase activities. These large subunits are the major products of the chloroplast genetic system; each circle of chloroplast DNA contains one gene for the large subunit, the mRNA being translated by free chloroplast ribosomes (Ellis, 1981) to produce a higher M_r precursor (Langridge, 1981). Since each chloroplast is polyploid, and each cell contains many chloroplasts, there are several hundred to several thousand genes for the large subunit in each cell.

The small subunit (M_r about 14 000) has no known specific function. In several eukaryotes the small subunit is encoded by a small multigene family in the nucleus (Berry-Lowe, McKnight, Shah & Meagher, 1982; Dunsmuir, Smith & Bedbrook, 1983; Broglie *et al.* 1983). The mRNA for the small subunit is translated by free cytoplasmic ribosomes to produce a higher M_r precursor possessing

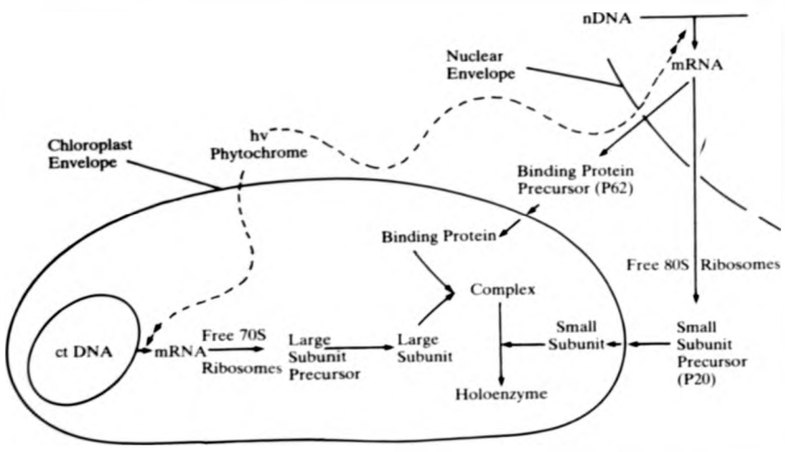


Fig. 1. Co-operation of nuclear and chloroplast genomes in the synthesis of Rubisco. The wavy dotted lines indicate that the stimulatory effect of light is mediated primarily at the level of transcription.

an aminoterminal extension of 40–60 amino acyl residues. This precursor is transported by an ATP-dependent post-translational mechanism across the chloroplast envelope, and the extension removed by a stromal metalloprotease (Ellis & Robinson, 1984). In some lower eukaryotes the small subunit is a product of the chloroplast genetic system so that protein transport is not involved in these species (Heinhorst & Shively, 1983).

One of the most striking features of Rubisco is its sheer abundance. Up to 65 % of the soluble protein in extracts of photosynthetic cells can be accounted for by this single enzyme. For this reason Rubisco has a good claim to be the most abundant protein on earth (Ellis, 1979). The reason for the abundance of Rubisco appears to be that it is such a sluggish catalyst that the organism has to synthesize many molecules in order to achieve the required rate of carboxylation. Since the rate of carboxylation restricts plant productivity in some cases, major efforts are being made to construct more efficient Rubisco molecules by mutagenesis of their cloned nucleotide sequences (Ellis & Gatenby, 1984). This abundance of Rubisco makes it ideal for molecular biological studies, and it is not too fanciful to regard it as the haemoglobin of the plant biochemist. Antibodies and cloned DNA hybridization probes are available for both subunits, and their use in the study of the synthesis of Rubisco is the subject of this article.

Besides the large and small subunits, another polypeptide appears to be involved in the synthesis of Rubisco (Fig. 1). The assembly of Rubisco from its subunits occurs in the stroma (Smith & Ellis, 1979), but attempts to dissociate Rubisco from higher plants into its subunits, and then to reassociate them with recovery of active enzymic activity, have so far been unsuccessful. Large subunits, prepared by treatment with detergents or high pH of Rubisco purified from higher plants, are insoluble in aqueous media, as are large subunits synthesized by *Escherichia coli* minicells containing inserted large subunit genes (Ellis & Gatenby, 1984). The holoenzyme however, is highly soluble, occurring at concentrations up to about 300 mg/ml in the stroma *in vivo*. Large subunits synthesized by isolated intact chloroplasts are also soluble, even though unassembled into holoenzyme (Blair & Ellis, 1973). This solubility is due to the binding of these newly synthesized large subunits to another stromal protein that we have termed the large subunit binding protein (Barraclough & Ellis, 1980; Ellis, 1981).

The large subunit binding protein is currently being studied at Warwick by S. Hemmingsen, S. D. Kung, C. Robinson and C. R. Lennox. The protein has been purified to homogeneity from *Pisum sativum* leaves, and sediments at a value of about 25S in the ultracentrifuge. Addition of 5 mM MgATP causes complete dissociation of the protein into a number of discrete lower relative molecular mass forms; removal of ATP reverses this dissociation. Analysis on denaturing polyacrylamide gels reveals a close doublet of subunit M_r about 60000, which gives a different proteolytic digestion pattern to the large subunit. The purified binding protein has been used to raise antisera in rabbits. These antisera show no cross reactivity with large subunits, and have been used to construct a quantitative assay for the binding protein by means of rocket immunoelectrophoresis (Fig. 2).

When *Pisum* leaf polysomes are run off in a wheat-germ protein-synthesizing system, the binding protein antisera precipitate a larger precursor of M_r about 62000. This precursor is taken up and processed by intact isolated *Pisum* chloroplasts. These observations are consistent with the view that the binding protein is encoded in the nucleus, and synthesized in the cytoplasm in precursor form prior to transport into the chloroplast.

Our working hypothesis is that the role of the large subunit binding protein is to maintain the newly-synthesized large subunits in soluble form prior to assembly into the holoenzyme. The failure to demonstrate the assembly of Rubisco from its subunits in a soluble system has so far precluded the rigorous testing of this hypothesis. Nevertheless, in view of the potentially obligatory role of this binding protein in the production of Rubisco, we are studying the effect of light on its synthesis during the greening of etiolated *Pisum* seedlings.

EXPRESSION OF RUBISCO GENES DURING GREENING

As noted in the Introduction, light is required for the completion of leaf development in higher plants and for the concomitant formation of mature,

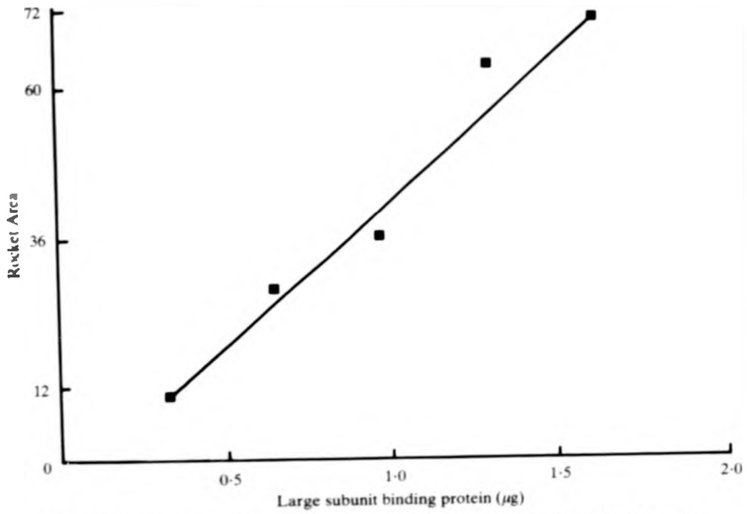


Fig. 2. Quantitative assay of the large subunit binding protein by rocket immunoelectrophoresis. Antisera raised against purified binding protein were used in rocket immunoelectrophoresis according to the procedure of Weeke (1973). Rockets were scanned and their areas determined by weighing. Binding protein was determined by the Biorad assay.

photosynthetically active chloroplasts. Our studies have centred on the greening of *Pisum sativum* seedlings grown in darkness for either 6 or 8 days. When these etiolated plants are transferred to continuous white light (100 μmoles m^{-2} s^{-1}, 400–700 nm) the rate of stem extension decreases markedly, and the leaf cells within the shoot apical buds begin to enlarge and divide. After 48 h of greening under these conditions the apical buds have nearly quadrupled in fresh weight, and their DNA content has increased about three-fold (G. I. Jenkins, M. R. Hartley & J. Bennett, unpublished), indicating a corresponding increase in cell number.

Some of the biochemical changes that accompany greening are shown in Fig. 3. There is an increase in the total protein content of the apical buds and in the amount of chlorophyll. Chlorophyll is formed from the protochlorophyllide which accumulates in darkness in a light-dependent reaction catalysed by the enzyme NADPH-protochlorophyllide-oxidoreductase (Griffiths, 1978). We have previously shown, using a radioimmune assay, that very little Rubisco is present in dark-grown plants but that this protein accumulates during greening, slowly at first, but then more rapidly from 36 to 48 h after exposure to light

(Jenkins *et al.* 1983; Bennett *et al.* 1984). Similar results have also been reported for *Pisum sativum* by Sasaki, Ishiye, Sakihama & Kamikubo (1981). The increase in the amount of Rubisco over the 48-h period is about 100-fold per apical bud; this increase clearly represents a considerable increase in the amount per leaf cell. Generally the amounts of the large and small subunits increase roughly in parallel during greening in white light in *Pisum sativum* (Sasaki *et al.* 1981; Jenkins *et al.* 1983; Bennett *et al.* 1984), and in other species (Walden & Leaver, 1981; Dean & Leech, 1982). This parallel accumulation of the two subunit types is quite remarkable considering the enormous difference in gene dosage referred

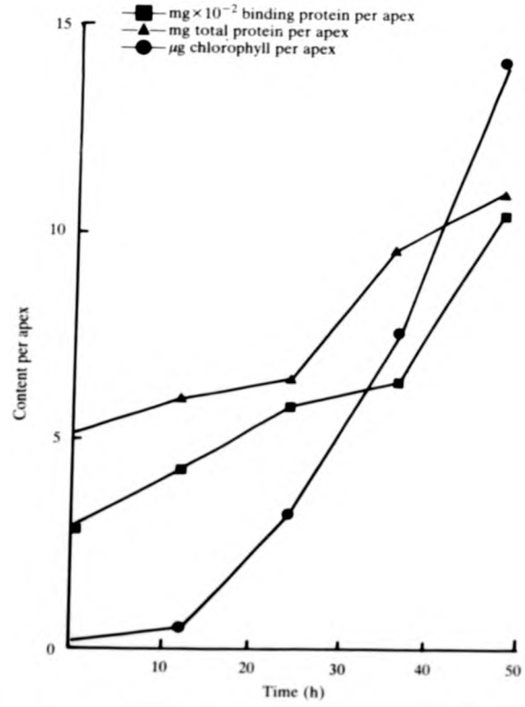


Fig. 3. Changes in the amounts of cellular components during greening. *Pisum* seedlings grown in darkness for 8 days were transferred to continuous white light and apices harvested over a 48 h period. Chlorophyll content was determined according to Arnon (1949); total protein was determined by the Biorad assay of extracts made in sodium dodecyl sulphate, and the large subunit binding protein quantified as in Fig. 2.

to earlier; some mechanism to achieve this co-ordination of expression of the large and small subunit genes must exist.

We have recently started to investigate changes in the abundance of the large subunit binding protein during greening, using the rocket immunoelectrophoresis assay (Fig. 2). As shown in Fig. 3, the binding protein is readily detectable in dark-grown apical buds, and accumulates steadily following illumination. One conclusion that can be drawn from these data is that the failure of Rubisco to accumulate in dark-grown *Pisum* seedlings is not due to a lack of binding protein needed for assembly into stable holoenzyme; some other explanation is required. A second conclusion is that the increase in the amount of binding protein during the 48 h greening period is only three- to four-fold, similar to the increase in cell number per apical bud estimated from measurements of DNA content. Thus the amount of binding protein per cell does not appear to change greatly, if at all, as a result of illumination, although further detailed measurements are required. It may be that light has no effect on the expression of the nuclear genes encoding the binding protein. Alternatively, both its rate of synthesis and its rate of breakdown could be stimulated by light, resulting in a constant level of the protein in each cell.

In our previous experiments we have attempted to define the levels at which light controls the expression of genes encoding Rubisco. Smith & Ellis (1982) demonstrated, by hybridization analysis using specific cloned DNA probes, that light induces an increase in the abundance of both large and small subunit mRNAs in total RNA extracts of *Pisum* apical buds. Subsequently we investigated whether the increase in mRNA contents, measured by quantitative dot-blot hybridization, is correlated with the accumulation of the large and small subunit polypeptides, measured by radioimmunoassay (Jenkins *et al.* 1984; Bennett *et al.* 1984). There is a very close correlation between the rate of accumulation of the small subunit polypeptide and its mRNA, but less so for the large subunit. Accumulation of the large subunit polypeptide lags behind that of its mRNA during greening, and less polypeptide is present in dark-grown plants than might be expected from its content of mRNA for the large subunit. Thus for the large, but not for the small, subunit polypeptide, it is necessary to propose that accumulation is controlled at some level other than the availability of mRNA transcripts for translation. The data (Fig. 3) do not support the suggestion that large subunits are synthesized in dark-grown seedlings and then degraded as a result of an insufficiency of the large subunit binding protein. It is possible that complexes of the large subunit with the binding protein are turned over in the absence of small subunit polypeptides or that other post-transcriptional controls influence the accumulation of the large subunit.

Information has been recently obtained regarding the photoreceptors which mediate the effect of light on the expression of the large and small subunit genes. In plants a number of different photoreceptors are believed to function in controlling development, the most extensively studied being phytochrome

(Pratt, 1982; Mohr & Shropshire, 1983). Phytochrome is a chromoprotein which exists in two photointerconvertible forms, one (P_r) absorbing maximally in the red (λmax 660 nm), and the other (P_{fr}) in the far-red (λmax 730 nm). Brief illumination with a low intensity of red light is sufficient to convert a large proportion of phytochrome into the P_{fr} form, which is regarded as the biologically active form in many responses, while illumination with far-red light converts P_{fr} back into P_r. This photoreversibility of phytochrome provides the basis of a simple test to establish its involvement in a given response; the inductive effect of a brief exposure to red light should be reversed by a subsequent far-red treatment, provided that insufficient time has elapsed between the light treatments for P_{fr} to initiate the train of events leading to the response in question. Application of this test has shown that phytochrome controls the expression of a number of plant genes (Jenkins, 1984).

In *Pisum* the expression of the small subunit genes is clearly under phytochrome control, although a brief red-light treatment produces only a small amount of the polypeptide (Jenkins *et al.* 1983) and hybridizable mRNA (Thompson *et al.* 1983; Jenkins *et al.* 1983) relative to that found under continuous white light. Phytochrome also controls the abundance of hybridizable small subunit mRNA in *Lemna gibba* (Stiekema *et al.* 1983). The situation with regard to the large subunit is more complex, since both red and far-red light treatments are equally effective in increasing the amount of both the large subunit polypeptide and its mRNA in 8-day-old dark-grown seedlings of *Pisum* (Jenkins *et al.* 1983). Similar results for large subunit mRNA have been reported for *Sinapsis alba* (Link, 1982). Phytochrome is probably involved, since no other known plant photoreceptor absorbs in the far red to any great extent; the most likely explanation is that even the small amount of P_{fr} formed in far-red light is sufficient to saturate the response for the large subunit, although not for the small. There are reports from other laboratories that red light is more effective than far-red at increasing the content of large subunit mRNA in *Pisum* (Thompson *et al.* 1983; Sasaki *et al.* 1983), but these experiments were performed on younger seedlings (5 days old), and this difference might be important.

We have recently started to investigate in greater detail the time course of small subunit mRNA accumulation during greening of etiolated *Pisum* seedlings. Fig. 4 shows the hybridization of a ^{32}P-labelled cloned cDNA probe specific for the small subunit gene to total RNA extracts of apical buds fractionated by agarose gel electrophoresis and then transferred to nitrocellulose. The content of small subunit mRNA in RNA from dark-grown apical buds is below the level of detection by this method; when measured by dot-blot hybridization the content is about 1 % of the amount present in RNA from seedlings after 48 h greening (Jenkins *et al.* 1983). An increase in the abundance of small subunit mRNA is observed after 6 h of illumination with continuous white light, but this is followed by an actual decrease in the detectable amount of mRNA prior to the final, substantial increase up to 48 h (Fig. 4). This slight transient increase in

Fig. 4. Hybridization analysis of the abundance of small subunit mRNA during greening of *Pisum* apical buds. Pea plants were grown in darkness for 8 days (a), and then transferred to continuous white light (100 μmoles $m^{-2} s^{-1}$; 400–700 nm) for either 6 h (b), 9 h (c), 12 h (d), 24 h (e), 36 h (f) or 48 h (g). Control plants were kept in darkness for 48 h (h). Total RNA was extracted from apical buds harvested at these times, fractionated by denaturing agarose gel electrophoresis and transferred to nitrocellulose filters (Jenkins *et al.* 1983). The immobilized RNA was hybridized to ^{32}P-labelled cDNA specific for the small subunit gene (Bedbrook *et al.* 1980). The arrow indicates the small subunit mRNA.

small subunit mRNA content is variable, both in its extent and its timing, but it is repeatedly observed. Possible mechanisms for this effect will be mentioned in the next section. It is clear however, that the effect of light on small subunit mRNA accumulation is more complex than we at first envisaged.

THE PHOTOREGULATION OF TRANSCRIPTION

The results discussed in the previous section demonstrate unequivocally that light induces an increase in the steady state concentrations of both small and large subunit mRNAs in *Pisum* apical buds, and that this increase is of major importance in accounting for the accumulation of the polypeptides during greening.

The question thus arises as to how this increase in mRNA content is produced. One possible explanation is that light increases the rates of transcription of the large and small subunit genes; another would be that the rate of mRNA synthesis is unchanged in the light, but that light causes a decrease in the rate of mRNA degradation. In order to distinguish between these possibilities for the small subunit genes we have undertaken measurements of small subunit gene transcription by nuclei isolated from *Pisum* apical buds (Gallagher & Ellis, 1982). Corresponding studies of large subunit gene transcription in isolated chloroplasts are now being initiated.

Nuclei are isolated from apical buds by Percoll density gradient centrifugation following homogenization. The nuclei incorporate labelled UMP, supplied as UTP, into RNA, which is heterodisperse in size up to about 25S. Fig. 5 shows that the incorporation proceeds linearly over the first 20 min of incubation, and then slowly decreases in rate. No incorporation is observed in the absence of nucleoside triphosphates. The addition of α-aminitin at 10 μg/ml, a concentration which inhibits completely transcription by RNA polymerase II, inhibits RNA synthesis by about 36%. Studies with other species have shown that transcription by

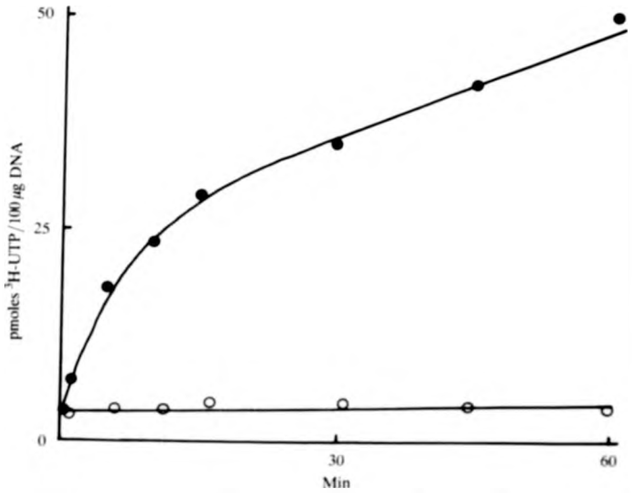


Fig. 5. Time course of RNA synthesis by isolated *Pisum* nuclei. Nuclei were incubated in a buffer containing 50 mM-tris-HCl, pH 7·8, 75 mM-NH_4Cl, 10 mM-$MgCl_2$, 20% glycerol, 500 μM-ATP, GTP & CTP, 10 μM [5,6-^{3}H]-UTP at 2 μCi/μl, and 10^6 nuclei (10 μg DNA) per 25 μl. At various times aliquots were removed and TCA-insoluble counts determined in duplicate. Symbols: ●, complete incubation mixture; ○, unlabelled nucleotides omitted.

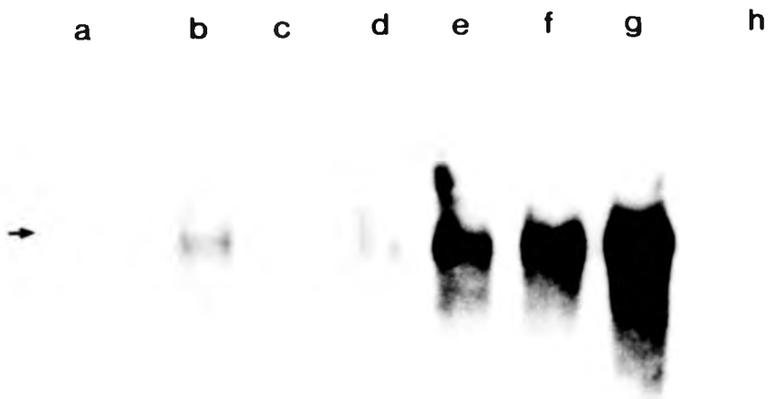


Fig. 4. Hybridization analysis of the abundance of small subunit mRNA during greening of *Pisum* apical buds. Pea plants were grown in darkness for 8 days (a), and then transferred to continuous white light (100 μmoles m^{-2} s^{-1}, 400–700 nm) for either 6 h (b), 9 h (c), 12 h (d), 24 h (e), 36 h (f) or 48 h (g). Control plants were kept in darkness for 48 h (h). Total RNA was extracted from apical buds harvested at these times, fractionated by denaturing agarose gel electrophoresis and transferred to nitrocellulose filters (Jenkins *et al.* 1983). The immobilized RNA was hybridized to ^{32}P-labelled cDNA specific for the small subunit gene (Bedbrook *et al.* 1980). The arrow indicates the small subunit mRNA.

small subunit mRNA content is variable, both in its extent and its timing, but it is repeatedly observed. Possible mechanisms for this effect will be mentioned in the next section. It is clear however, that the effect of light on small subunit mRNA accumulation is more complex than we at first envisaged.

THE PHOTOREGULATION OF TRANSCRIPTION

The results discussed in the previous section demonstrate unequivocally that light induces an increase in the steady state concentrations of both small and large subunit mRNAs in *Pisum* apical buds, and that this increase is of major importance in accounting for the accumulation of the polypeptides during greening.

The question thus arises as to how this increase in mRNA content is produced. One possible explanation is that light increases the rates of transcription of the large and small subunit genes; another would be that the rate of mRNA synthesis is unchanged in the light, but that light causes a decrease in the rate of mRNA degradation. In order to distinguish between these possibilities for the small subunit genes we have undertaken measurements of small subunit gene transcription by nuclei isolated from *Pisum* apical buds (Gallagher & Ellis, 1982). Corresponding studies of large subunit gene transcription in isolated chloroplasts are now being initiated.

Nuclei are isolated from apical buds by Percoll density gradient centrifugation following homogenization. The nuclei incorporate labelled UMP, supplied as UTP, into RNA, which is heterodisperse in size up to about 25S. Fig. 5 shows that the incorporation proceeds linearly over the first 20 min of incubation, and then slowly decreases in rate. No incorporation is observed in the absence of nucleoside triphosphates. The addition of α-aminitin at 10 μg ml, a concentration which inhibits completely transcription by RNA polymerase II, inhibits RNA synthesis by about 36 %. Studies with other species have shown that transcription by

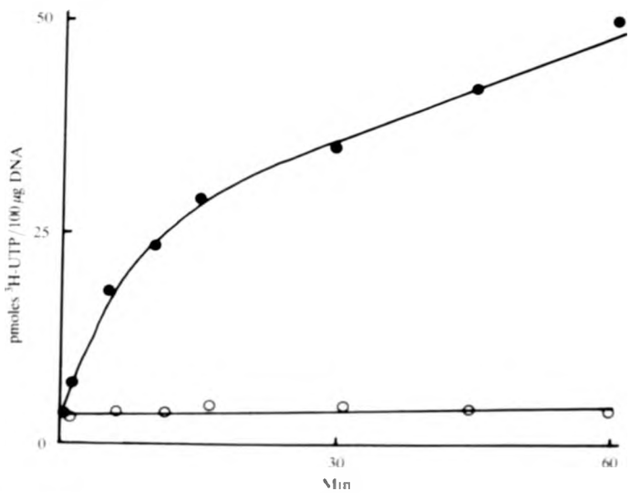


Fig. 5. Time course of RNA synthesis by isolated *Pisum* nuclei. Nuclei were incubated in a buffer containing 50 mM-tris-HCl, pH 7·8, 75 mM-NH_4Cl, 10 mM-$MgCl_2$, 20 % glycerol, 500 μM-ATP, GTP & CTP, 10 μM [5,6-^{3}H]-UTP at 2 μCi/μl, and 10^6 nuclei (10 μg DNA) per 25 μl. At various times aliquots were removed and TCA-insoluble counts determined in duplicate. Symbols: ●, complete incubation mixture; ○, unlabelled nucleotides omitted.

isolated nuclei represents elongation of already engaged polymerases rather than the combined result of elongation and initiation (Tsai *et al.* 1978; Dermann *et al.* 1981). Thus measurement of small subunit gene transcription in isolated nuclei can be used to distinguish between changes in either transcription or mRNA turnover as mechanisms which regulate the steady state amount of small subunit mRNA.

Gallagher & Ellis (1982) used cloned cDNA hybridization probes to detect small subunit gene transcripts in the labelled RNA synthesized by isolated nuclei. Small subunit transcripts could barely be detected in the RNA products of nuclei obtained from dark-grown apical buds; DNA-excess hybridization showed that the transcripts were 18 times more abundant in RNA synthesized by nuclei isolated from light-grown buds. Although these experiments suggest that small subunit transcripts are synthesized at a greater rate in nuclei isolated from illuminated plants, it is possible that there is no dark–light difference in the rate of transcription, but that newly synthesized transcripts are rapidly degraded in nuclei from dark-grown plants. To test this possibility Gallagher & Ellis (1982) undertook a pulse-chase experiment with the isolated nuclei. Nuclei from dark-grown buds were incubated with ^{32}P-UTP for 10 min, and aliquots incubated for a further period in the presence of excess unlabelled UTP and actinomycin D. The low amount of small subunit transcript synthesized during the initial 10 min pulse persisted during the chase period for at least 120 min, indicating the stability of the transcripts. Thus there is strong evidence that the increase in the small subunit mRNA content during greening in *Pisum* is the result of transcriptional control.

Our aim is to understand how a light signal detected by phytochrome, and possibly other photoreceptors, is able to effect a change in transcriptional activity. To do this it is necessary to develop an *in vitro* transcriptional system in which the action of light can be reproduced over a short period. In an attempt to find rapid effects of light we have monitored the rate of transcription of small subunit genes in isolated nuclei following the transfer of dark-grown plants to continuous white light. The results (Fig. 6) correspond well with those for the accumulation of the small subunit mRNA during greening (Fig. 4); that is, the rate of transcription is initially very low, and increases slowly to a maximum after about 36 h illumination. Moreover, a transient increase in the rate of small subunit gene transcription is observed within 1 h of exposure to light, which is consistent with the transient increase in small subunit mRNA content during the early stages of greening (Fig. 4).

A number of models for the control of transcription of specific genes have been proposed. These include the methylation of non-expressed genes (Razin & Riggs, 1980) and alterations in chromatin configuration (Weintraub & Goudine, 1976). It is also possible that various members of the small subunit multigene family are controlled differently by light. Our present speculation is that initiation occurs in dark-grown plants, but only slowly, and that illumination causes

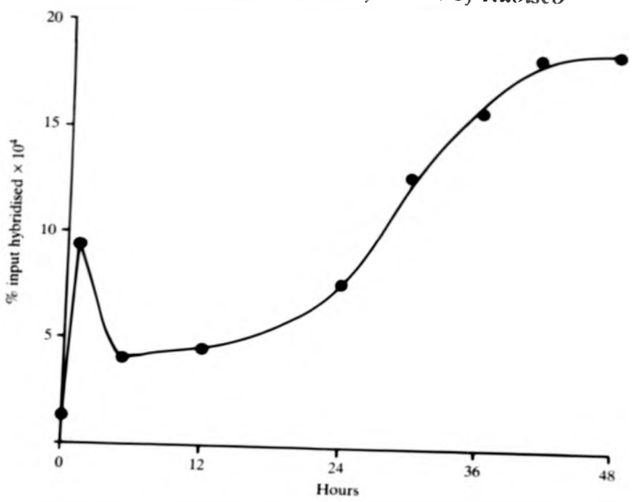


Fig. 6. Changes in the rate of transcription of small subunit genes during greening of *Pisum* seedlings. Pea plants were grown from seed in darkness for 6 days and then transferred to continuous white light as in Fig. 4. Apices were harvested and nuclei isolated at various times over a 48 h period. The rate of transcription of small subunit genes was determined as described by Gallagher & Ellis (1982).

engaged polymerases to produce a small amount of small subunit transcript; a relatively long period of illumination is then required before re-initiation is completed, and transcription can proceed at its maximal rate. It is also evident from Fig. 4 that the small subunit transcripts produced initially are unstable and subject to degradation.

Although this initial burst of transcription occurs rapidly following illumination, it is too small and variable to provide the basis of an *in vitro* analysis. We have found however, that large rapid changes in small subunit gene transcription are observed if plants which are capable of a high rate of small subunit transcription are subjected to brief light–dark transitions. As shown in Fig. 7, when competent plants are transferred to darkness the rate of transcription decreases to a low level within 20 min. The rate of transcription then rapidly increases over a similar time period if these dark-treated plants are returned to continuous white light. It should be noted that the initial stages of nuclear isolation from dark-treated plants are carried out in darkness.

We are at present attempting to identify the photoreceptors which mediate these different effects of light; that is, the rapid initial increase in transcription,

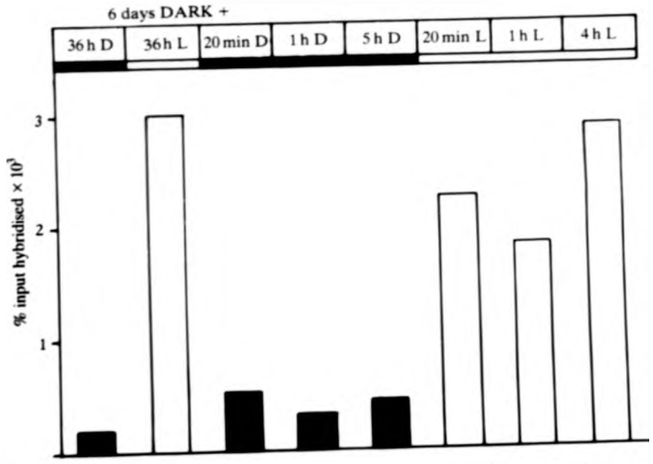


Fig. 7. Rapid light-induced changes in the rate of transcription of *Pisum* small subunit genes. Pea plants were grown in darkness for 6 days, transferred to continuous white light (see Fig. 4) for 36 h, and returned to darkness for either 20 min, 1 h or 5 h. Plants left in darkness for 5 h were then transferred to white light for either 20 min, 1 h or 4 h. Apical buds were harvested from the plants at these times and nuclei were isolated. The harvesting and initial stages of isolation of the nuclei were performed in darkness. The rate of transcription of small subunit genes was determined as described by Gallagher & Ellis (1982).

the slow development of the competence to transcribe rapidly and lastly, the rapid switch induced by a dark–light transition. We hope that these studies will provide information that will lead to the development of a biochemical analysis of the molecular basis of the photoregulation of transcription of the small subunit genes.

REFERENCES

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* **24**, 1–15.

Barraclough, R. & Ellis, R. J. (1980). Protein synthesis in chloroplasts IX. Assembly of newly-synthesized large subunits into ribulose bisphosphate carboxylase in isolated intact pea chloroplasts. *Biochim. Biophys. Acta* **608**, 19–31.

Bedbrook, J. R., Smith, S. M. & Ellis, R. J. (1980). Molecular cloning and sequencing of DNA encoding the small subunit of chloroplast ribulose-1,5-bisphosphate carboxylase. *Nature* **287**, 692–697.

Bennett, J., Jenkins, G. I. & Hartley, M. R. (1984). Differential regulation of the accumulation of the light-harvesting chlorophyll a/b-binding complex and ribulose bisphosphate carboxylase/oxygenase in greening pea leaves. *J. cell. Biochem.* (in press).

Berry-Lowe, S., McKnight, T. D., Shah, D. M. & Meagher, R. B. (1982). The nucleotide sequence, expression, and evolution of one member of a multigene family encoding the small subunit of ribulose-1,5-bisphosphate carboxylase in soybean. *J. molec. Appl. Genet.* **1**, 483–498.

Blair, G. E. & Ellis, R. J. (1973). Protein synthesis in chloroplasts I. Light-driven synthesis of the large subunit of Fraction 1 protein by isolated pea chloroplasts. *Biochim. Biophys. Acta* **319**, 223–234.

Bradbeer, J. W. (1981). Development of photosynthetic function during chloroplast biogenesis. In *The Biochemistry of Plants* (eds N. K. Boardman & M. D. Hatch), vol. 8, pp. 424–472. New York: Academic Press.

Broglie, R., Coruzzi, G., Lamppa, G., Keith, B. & Chua, N.-H. (1983). Structural analysis of nuclear genes coding for the precursor to the small subunit of wheat ribulose-1,5-biphosphate carboxylase. *Biotechnology* **1**, 53–61.

Coen, D. M., Bedbrook, J. R., Bogorad, L. & Rich, A. (1977). Maize chloroplast DNA encoding the large subunit of ribulose bisphosphate carboxylase. *Proc. natn. Acad. Sci., U.S.A.* **74**, 5487–5491.

Dean, C. & Leech, R. M. (1982). The co-ordinated synthesis of the large and small subunits of ribulose bisphosphate carboxylase during early cellular development within a seven-day old wheat leaf. *FEBS Letts.* **140**, 113–116.

Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray, M. & Darnell, J. E. (1981). Transcriptional control in the production of liver-specific mRNAs. *Cell* **23**, 731–739.

Dunsmuir, P., Smith, S. M. & Bedbrook, J. R. (1983). A number of different nuclear genes for the small subunit of RuBPCase are transcribed in petunia. *Nucl. Acids Res.* **11**, 4177–4183.

Ellis, R. J. (1979). The most abundant protein in the world. *Trends in Biochem. Sci.* **4**, 241–244.

Ellis, R. J. (1981). Chloroplast proteins: synthesis, transport and assembly. *Ann. Rev. Plant Physiol.* **32**, 111–137.

Ellis, R. J. (1983). Chloroplast protein synthesis: principles and problems. In *Subcellular Biochemistry* vol. 9, (ed. D. B. Roodyn), pp. 237–261. New York: Plenum Publishing Co.

Ellis, R. J. & Gatenby, A. A. (1984). Ribulose bisphosphate carboxylase: properties and synthesis. In *The Genetic Manipulation of Plants and its Application to Agriculture. Ann. Proc. Phytochem. Soc. Eur.* (eds P. Lea & G. R. Stewart), Oxford University Press (in press).

Ellis, R. J. & Robinson, C. (1984). Post-translational transport and processing of cytoplasmically-synthesized precursors of organellar proteins. In *The Enzymology of the Post-Translational Modification of Proteins* (eds R. B. Freedman & H. C. Hawkins), Vol. II. New York: Academic Press (in press).

Gallagher, T. F. & Ellis, R. J. (1982). Light-stimulated transcription of genes for two chloroplast polypeptides in isolated pea leaf nuclei. *The EMBO J.* **1**, 1493–1498.

Gallagher, T. F., Jenkins, G. I., Smith, S. M. & Ellis, R. J. (1984). Photoregulation of the nuclear and chloroplast genes for ribulose bisphosphate carboxylase. In *Chloroplast Biogenesis* (ed. R. J. Ellis), Seminar Series No. 21 of the Society for Experimental Biology. Cambridge University Press (in press).

Griffiths, W. T. (1978). Reconstitution of chlorophyllide formation by isolated chloroplast membranes. *Biochem. J.* **174**, 681–692.

Heinhorst, S. & Shively, J. M. (1983). Encoding of both subunits of ribulose-1,5-bisphosphate carboxylase by the organelle genome of *Cyanophora paradoxa*. *Nature* **304**, 373–374.

Jenkins, G. I. (1984). The photoregulation of gene expression in plants. In *Oxford Surveys of Plant Molecular and Cell Biology* (ed. B. J. Miflin). Oxford University Press (in press).

Jenkins, G. I., Gallagher, T. F., Hartley, M. R., Bennett, J. & Ellis, R. J. (1984). Photoregulation of gene expression during chloroplast biogenesis. In *Advances in Photosynthesis Research* (ed. C. Sybesma), Vol IV. pp.863–872. The Hague: Junk.

Jenkins, G. I., Hartley, M. R. & Bennett, J. (1983). Photoregulation of chloroplast development: transcriptional, translational and post-translational controls? *Phil. Trans. R. Soc. London B* **303**, 419–431.

KIRK, J. T. O. & TILNEY-BASSETT, R. A. E. (1978) *The Plastids, their Chemistry, Growth, Structure and Inheritance*. Second ed. Amsterdam: Elsevier North-Holland.

LANGRIDGE, P. (1981) Synthesis of the large subunit of spinach ribulose bisphosphate carboxylase may involve a precursor polypeptide. *FEBS Letts* **123**, 85–89.

LISK, G. (1982) Phytochrome control of plastid mRNA in mustard. *Planta* **154**, 81–86.

LORIMER, G. (1981) The carboxylation and oxygenation of ribulose-1,5-bisphosphate: the primary events in photosynthesis and photorespiration. *Ann. Rev. Plant Physiol.* **32**, 349–383.

MIZIORKO, H. M. & LORIMER, G. (1983) Ribulose-1,5-bisphosphate carboxylase–oxygenase. *Ann. Rev. Plant Physiol.* **52**, 507–535.

MOHR, H. & SHROPSHIRE, W. JR. (1983) An introduction to photomorphogenesis for the general reader. In *Encyc. Plant Physiol.* New Series Vol. 16A (eds W. Shropshire & H. Mohr), pp. 24–38. Berlin: Springer.

PRATT, L. H. (1982) Phytochrome: the protein moiety. *Ann. Rev. Plant Physiol.* **33**, 557–582.

RAZIS, A. & RIGGS, A. D. (1980) DNA methylation and gene expression. *Science* **210**, 604–610.

SASAKI, Y., ISHIYE, M., SAKIHAMA, T. & KAMIKUBO, T. (1981) Light-induced increase in mRNA activity coding for the small subunit of ribulose-1,5-bisphosphate carboxylase. *J. biol. Chem.* **256**, 2315–2320.

SASAKI, Y., SAKIHAMA, T., KAMIKUBO, T. & SHINOZAKI, K. (1983) Phytochrome-mediated regulation of two mRNAs, encoded by nuclei and chloroplasts, of ribulose-1,5-bisphosphate carboxylase-oxygenase. *Eur. J. Biochem.* **133**, 617–620.

SMITH, S. M. & ELLIS, R. J. (1979) Processing of small subunit precursor of ribulose bisphosphate carboxylase and its assembly into whole enzyme are stromal events. *Nature* **278**, 662–664.

SMITH, S. M. & ELLIS, R. J. (1981) Light-stimulated accumulation of transcripts of nuclear and chloroplast genes for ribulose bisphosphate carboxylase. *J. molec. Appl. Genet.* **1**, 127–137.

STIEKEMA, W. J., WIMPEE, C. F., SILVERTHORNE, J. & TOBIN, E. M. (1983) Phytochrome control of the expression of two nuclear genes encoding chloroplast proteins in *Lemna gibba*. *Plant Physiol.* **72**, 717–724.

THOMPSON, W. F., EVERETT, M., POLANS, N. O., JORGENSEN, R. A. & PALMER, J. D. (1983) Phytochrome control of RNA levels in developing pea and mung bean leaves. *Planta* **158**, 487–500.

TOBIN, E. M. (1981) Phytochrome-mediated regulation of messenger RNAs for the small subunit of ribulose-1,5-bisphosphate carboxylase and the light-harvesting chlorophyll a/b binding protein in *Lemna gibba*. *Plant molec. Biology* **1**, 35–51.

TSAI, S. Y., ROOP, D. R., TSAI, M. J., STEIN, J. P., MEANS, A. R. & O'MALLEY, B. W. (1978) Effect of estrogen on gene expression in the chick oviduct. Regulation of the ovomucoid gene. *Biochemistry* (Wash.) **17**, 5773–5780.

WALDEN, R. & LEAVER, C. J. (1981) Synthesis of chloroplast proteins during germination and early development of cucumber. *Plant Physiol.* **67**, 1090–1096.

WEEKE, B. (1973) In *A Manual of Quantitative Electrophoresis. Methods and Applications* (eds N. H. Alexsen, J. Kroll & B. Weeke). *Scand. J. Immunol.* vol. 2, supplement no. 1. Publishers.

WEINTRAUB, H. & GROUDINE, M. (1976) Chromosomal subunits in active genes have an altered conformation. *Science* **193**, 848–856.

The carboxylase large subunit binding protein: photoregulation and reversible dissociation

C.RUTH LENNOX and R.JOHN ELLIS
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K.

Biochem. Soc. Trans., in press.

The carboxylase large subunit binding protein: photoregulation and reversible dissociation

C.RUTH LENNOX and R.JOHN ELLIS
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K.

Biochem. Soc. Trans., in press.

Chloroplasts contain an abundant soluble protein that binds in a non-covalent manner to newly-synthesized large subunits of the photosynthetic CO_2-fixing enzyme ribulose-1, 5-bisphosphate carboxylase-oxygenase or RuBP carboxylase (Barraclough & Ellis, 1980; Roy et al. 1982). The large subunits of RuBP carboxylase are synthesized within the chloroplast, while the small subunits are synthesized in precursor form by cytoplasmic ribosomes, and are subsequently transported across the chloroplast envelope before combination with large subunits in the stromal compartment (Ellis, 1981). Large subunits of RuBP carboxylase from higher plants are insoluble in aqueous media, both when isolated from the holoenzyme and when synthesized from cloned genes in Escherichia coli (Gatenby, 1984). The proposed role of the binding protein is to maintain the carboxylase large subunits in soluble form suitable for assembly with small subunits (Barraclough & Ellis, 1980). Consistent with this proposal is the observation that large subunits newly-syntnesized by isolated chloroplasts will transfer from the binding protein to the carboxylase holoenzyme on treatment of stromal extracts with MgATP (Milos & Roy, 1984; Milos et al. 1985). However it has not been established that combination of large subunits with the binding protein is an obligatory step in the assembly of RuBP carboxylase. A precedent for such an obligatory assembly protein is nucleoplasmin, which is required for the correct assembly of nucleosomes from DNA and histones in Xenopus extracts (Lasky & Earnshaw, 1980).

If such an obligatory assembly role for the large subunit binding protein exists, it will be necessary to express its cloned genes in Escherichia coli, so that attempts to produce improved mutant forms of higher plant RuBP carboxylase by genetic engineering techniques can progress (Ellis & Gatenby, 1984).

The large subunit binding protein of Pisum sativum chloroplasts has been purified to homogeneity and characterized (Hemmingsen & Ellis, submitted). The purified protein has a relative molecular mass of 720,000 and is composed of equal numbers of two types of subunits of relative molecular mass 61,000 and 60,000 respectively. These subunits have different aminoterminal sequences, there being only two positions in the first 30 residues where the same amino acids occur. Neither sequence corresponds to that of the RuBP carboxylase large subunit. The binding protein subunits are not synthesized by isolated chloroplasts, but a higher molecular mass form is immunoprecipitated from products of Pisum sativum polysomes translated in a wheat-germ extract. These data indicate that the binding protein is a product of cytoplasmic ribosomes and that its two subunits are probably encoded in nuclear genes. Polyclonal antisera raised against purified large subunit binding protein do not cross-react with RuBP carboxylase large subunits. Immunoblotting reveals the presence of binding protein in extracts of tobacco, wheat and barley leaves, and in extracts of plastids isolated from castor bean endosperm. In this paper we report that the

accumulation of binding protein in Pisum sativum leaves is stimulated by light, and that the effect of MgATP is to promote a reversible dissociation to monomeric subunits.

The accumulation of RuBP carboxylase by growing Pisum leaves is strongly stimulated by light, an effect mediated primarily by an increase in transcription (Gallagher & Ellis, 1982). The effect of light on the accumulation of the large subunit binding protein was studied in two types of experiment. In the first, pea seeds were germinated in total darkness for 8 days, and then exposed to continuous white light for up to 48 h; in the second, pea plants raised from seed for 8 days in total darkness were compared with pea plants raised from seed under a 12 h photoperiod for 8 days. Soluble extracts of the apical buds were made, and the content of RuBP carboxylase and binding protein determined by rocket immunoelectrophoresis. Fig.1 shows that dark-grown plants contain detectable amounts of both proteins but that the amounts per apical bud increase when these dark-grown plants are illuminated. The main increase occurs after 24 h illumination, and the increase in the amount of carboxylase is greater than the increase in the amount of binding protein. No increase occurs in either protein if the dark-grown plants are maintained in darkness. Comparison of dark-grown apical buds with light-grown apical buds shows that the increase in binding protein content due to light is similar in magnitude (5 to 7-fold) to the increase in fresh weight and total soluble protein (Table 1). However the increase in

RuBP carboxylase protein due to light is much greater (30-fold), confirming previous results (Smith & Ellis, 1981). The small amount of RuBP carboxylase protein present in dark-grown apical buds has the same specific activity as that present in light-grown buds (Table 1). We conclude from these results that the accumulation of the large subunit binding protein is stimulated by light, but to a much lesser degree than the accumulation of RuBP carboxylase.

The dissociation of the binding protein by MgATP reported by Bloom et al (1983) was further studied by means of electrophoresis on non-denaturing gradient polyacrylamide gels. Such gels exhibit a much greater resolving power than the non-denaturing gels used in the discovery of the binding protein (Barraclough & Ellis, 1980), and allow the detection of specific proteins by immunoblotting. Fig.2 shows that the treatment of stromal extracts with concentrations of MgATP as low as 0.1 mM results in a partial conversion of the 720,000 M_r form of the binding protein to the monomeric form of M_r 60,000. This conversion increases with increasing concentrations of MgATP, but is not complete even at 5 mM. It is important to note that some monomer is present even in stromal extracts which have been dialysed to remove endogenous MgATP (Fig.2, track 1). Removal of added ATP by protein synthesis results in reformation of the oligomeric form from the monomeric form (Fig.2, tracks 6-8).

These observations suggest that the binding protein undergoes a reversible dissociation between the oligomeric

form and the monomeric form, MgATP causing the equilibrium to shift towards the monomeric form. This equilibrium can be represented as follows:-

$$\alpha_6\beta_6 \underset{\text{MgATP}}{\rightleftharpoons} 6\alpha + 6\beta$$

where α and β represent the two types of subunit. Under *in vivo* conditions, the bulk of the binding protein will be in the monomeric form, since the ATP concentration in the stroma is greater than 1 mM (Hampff *et al*, 1982). When a stromal extract is prepared, the ATP concentration is reduced by a factor of at least 100-fold, resulting in a shift to the oligomeric form to which newly-synthesized carboxylase large subunits are bound. Whether these large subunits are also bound to the monomeric form of the binding protein, and whether either form is involved in the assembly of RuBP carboxylase, remains to be elucidated.

C.R.L. was supported by a postgraduate award from the Science and Engineering Research Council.

Barraclough,R. & Ellis, R.J. (1980) Biochim.Biophys.Acta 608, 19-31

Blair,G.E. & Ellis,R.J. (1973) Biochim.Biophys.Acta 319, 223-234

Bloom,M.V., Milos,P. & Roy,H. (1983) Proc.Natl.Acad.Sci.USA 80, 1013-1017

Bradford,M.M. (1976) Anal.Biochem. 72, 248-254

Burnette,W.N. (1981) Anal.Biochem. 112, 195-203

Ellis,R.J. (1981) Ann.Rev.Plant Physiol. 32, 111-137

Ellis, R.J. & Gatenby,A.A. (1984) in The Genetic Manipulation of Plants and its Application to Agriculture (Lea,P.J. & Stewart,G.R. eds.), Ann.Proc.Phytochemical Soc.Eur. vol.23, 41-60

Gatenby,A.A.(1984) Eur.J.Biochem. 144, 361-366

Hampff,R., Goller,M. & Ziegler,H. (1982) Plant Physiol. 69, 448-455

Lasky,R.A. & Earnshaw,W.C. (1980) Nature (Lond.) 286, 763-767

Laurell,C. (1966) Anal.Biochem. 15, 45-52

Lorimer,G.H., Badger,M.R. & Andrews, T.J. (1977) Anal.Biochem. 78,66-75

Milos,P., Bloom,M. & Roy,H. (1985) Plant Mol.Biol.Reporter 3, 33-42

Milos,P. & Roy,H. (1984) J.Cell.Biochem. 24, 153-162

Roy,H., Bloom,M., Milos,P. & Monroe,M. (1982) J.Cell Biol. 94, 20-27

Smith,S.M. & Ellis,R.J. (1981) J.Mol.Appl.Genet. 127-137

Table 1. Comparison of RuBP carboxylase and large subunit binding protein contents in dark-grown and light-grown pea plants

Pea seeds were germinated either in complete darkness at 20°C or under warm-white fluorescent lights (50 μmoles $m^{-2}s^{-1}$, 12 h photoperiod) at 20°C for 8 days. Apical buds were excised, weighed and the total soluble protein extracted as described in Fig.1. The amount of protein present was estimated by the dye-binding method of Bradford (1976). The amounts of RuBP carboxylase and binding protein present in the extracts were determined by rocket immunoelectrophoresis. The activity of RuBP carboxylase was determined by the method of Lorimer et al (1977).

	Amount per apical bud (mg)				Specific activity of RuBP carboxylase
Growth condition	Fresh weight	Soluble protein	RuBP carboxylase	Binding protein	(nmols/min per mg carboxylase)
Darkness	22	0.8	0.11	0.014	50
Light	108	4.0	3.32	0.097	47

Figure legends

Fig.1. Light-stimulated accumulation of RuBP carboxylase and the large subunit binding protein

Pea plants (Pisum sativum var.Feltham First) were grown from seed either in total darkness at 20°C for up to 10 days or grown in total darkness at 20°C for 8 days and then exposed to continuous white light (200 μmoles $m^{-2}s^{-1}$) for up to 48 h at 20°C. Apical buds were excised and frozen in liquid nitrogen. Total soluble protein was extracted by the method of Smith & Ellis (1981). The amounts of RuBP carboxylase and the large subunit binding protein present in each extract were determined by rocket immunoelectrophoresis as described by Laurell (1966). Symbols: ●, large subunit binding protein; ■, RuBP carboxylase. Symbols in brackets refer to extracts from plants maintained in darkness.

Fig.2. Reversible dissociation of the carboxylase large subunit binding protein by ATP

Pea plants were grown under a 12 h photoperiod for 10 days and chloroplasts extracted as described by Blair & Ellis (1973). Washed chloroplasts were lysed by resuspension in 10 mM Tris-HCl, 10 mM $MgSO_4$, pH 8.0. Membranes were removed by centrifugation and the supernatant extract dialysed against fresh lysis buffer at 4°C. Aliquots of dialysed extract were incubated at 0°C with various concentrations of ATP for 1 h. Other aliquots were incubated with 2 mM ATP, 0.2 mM GTP and 80 mM KCl at 27°C for up to 120 min to allow protein synthesis by free chloroplast ribosomes to remove the added ATP. All aliquots were then analyzed by electrophoresis under non-denaturing conditions on a 4-30% (w/v) gradient polyacrylamide gel.

After electrophoresis the proteins were transferred onto a nitrocellulose sheet by electroblotting (Burnette, 1981) and the positions of the binding protein determined by incubating the sheet with antibody to the binding protein and ^{125}I-labelled protein A. Molecular weights were estimated by marker proteins. The figure shows an autoradiograph of the nitrocellulose sheet.
Track 1, no ATP; track 2, 0.1 mM ATP; track 3, 0.5 mM ATP; track 4, 1.0 mM ATP; track 5, 5.0 mM ATP. Tracks 6,7 and 8 show the results of incubating the stromal extracts under conditions that permit protein synthesis for 60, 90 and 120 min respectively.
Arrows: A, binding protein oligomer of M_r 720,000; B, binding protein monomer of M_r 60,000.

Fig. 1

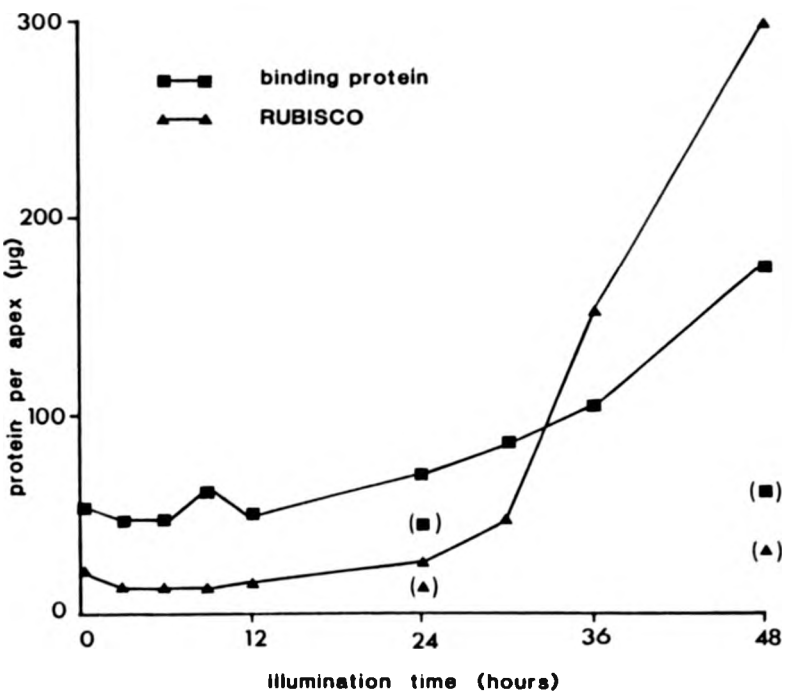

300
200
100
0
protein per apex (µg)
binding protein
RUBISCO
(■)
(▲)
(■)
(▲)
0
12
24
36
48
illumination time (hours)

Fig. 2